specification of the present application, *see, e.g.*, page 9, lines 1-9, page 16, line 9-24, page 20, lines 30-33, page 23, line 30 to page 25, line 35, and page 40, line 17 to page 44, line 4, and do not constitute new subject matter. Claims 41-57 and 71-78 will, therefore be pending upon entry of this Amendment. A copy of the claims which will be pending upon entry of this Amendment is attached hereto as Exhibit B.

Entry of the foregoing amendments and consideration of these remarks are respectfully requested.

THE REJECTION UNDER 35 U.S.C. § 103 SHOULD BE WITHDRAWN

Claims 41-57 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Mitsuhashi *et al.* (U.S. Patent No. 4,659,569; "Mitsuhashi") and Sasaki *et al.* (JP 59-29831; "Sasaki"). The Examiner contends that Mitsuhashi and Sasaki teach eggs infected with viruses, particularly influenza virus, and that embryonated eggs less than ten days old are susceptible to virus infection and replication. Although the Examiner appreciates that neither of the cited references teach the "specific viral strains" of the claimed invention, according to the Examiner, it would have been *prime facie* obvious to one of ordinary skill in the art at the time of the invention, to apply the teachings of the cited references to make the composition of the claimed invention. According to the Examiner, one skilled in the art would have been motivated to do so because "it is notoriously old and well known in the art to propagate viruses in such eggs" and one would "reasonably expect the infected egg to yield replicated virus" (page 4 of the Office Action). For the reasons detailed below, however, Applicants respectfully assert that the rejection under 35 U.S.C. § 103(a) is in error and should be withdrawn.

A finding of obviousness requires a determination of the scope and content of the prior art, the level of ordinary skill in the art, the differences between the claimed subject matter and the prior art, and whether the differences are such that the subject matter as a whole would have been obvious to one of ordinary skill in the art at the time the invention was made. *Graham v. Deere* 383 U.S. 1 (1996). The proper inquiry is whether the art suggests the invention, and whether the art provides one of ordinary skill in the art *with a reasonable expectation of success*. *In re O'Farrell* 853 F.2d 894, 7 U.S.P.Q. 2d 1673 (Fed. Cir. 1988). Both the suggestion *and* the reasonable expectation of success must be founded in the prior art and not in the Appellants' disclosure. *In re Vaeck* 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed. Cir. 1991).

None of the cited references, alone or in combination teach or suggest the claimed invention, *i.e.*, a composition comprising an embryonated egg less than ten days old containing a recombinantly engineered attenuated negative strand RNA virus, *e.g.*, an influenza virus, with an impaired interferon antagonist phenotype. The cited references do not teach, suggest, or provide a motivation to one skilled in the art to produce a composition comprising an embryonated egg less than ten days old containing a recombinantly engineered attenuated negative strand RNA virus with an impaired interferon antagonist phenotype. Further, the cited references fail to teach, suggest, or provide a motivation to one skilled in the art to produce a composition comprising an embryonated egg less than ten days old containing a recombinantly engineered attenuated influenza virus having a mutation in the NS1 gene that diminishes or eliminates the ability of the NS1 gene product to antagonize the cellular interferon response.

Contrary to the Examiner's contention, neither Mitsuhashi nor Sasaki teach or suggest the claimed invention. Mitsuhashi relates to a process for the production of a viral vaccine comprising covalently attaching the virus to a saccharide to form a virus-saccharide conjugate. The vaccines produced in accordance with Mitsuhashi are to produce a higher level of immunoglobulin G and M, while producing little or no immunoglobulin E. There is no recognition, suggestion, or teaching in Mitsuhashi that the methods proposed are effective in embryonated eggs of a particular age, let alone immature embryonated eggs less than ten days old. In fact, the working examples provided in Mitsuhashi indiscriminately use different aged eggs, *e.g.*, in Examples 4 and 5, 10-day old embryonated eggs are used and in Example 7, 8-day old embryonated eggs are used). Mitsuhashi does not teach, suggest or provide a motivation to produce the composition of the claimed invention, *i.e.*, an embryonated egg less than ten days old containing a recombinantly engineered attenuated negative strand RNA virus with an impaired interferon antagonist phenotype. Further, Mitsuhashi does not teach, suggest or provide a motivation to produce a composition comprising an embryonated egg less than ten days old containing a recombinantly engineered attenuated influenza virus, let alone a recombinantly engineered attenuated influenza virus having a mutation in the NS1 gene that diminishes or eliminates the ability of the NS1 gene product to antagonize the cellular interferon response.[1]

Sasaki relates to a vaccine for pigs, obtained my adding Macrogol to an influenza virus that is propagated in allantoic cavity of a grown hen's egg of 9-11 days of age. Sasaki does not teach, suggest or provide a motivation to produce the composition of the claimed

invention, *i.e.*, an embryonated egg less than ten days old containing a recombinantly engineered attenuated negative strand RNA virus with an impaired interferon antagonist phenotype. Further, Sasaki does not teach, suggest or provide a motivation to produce a composition comprising an embryonated egg less than ten days old containing a recombinantly engineered attenuated influenza virus, let alone a recombinantly engineered attenuated influenza virus having a mutation in the NS1 gene that diminishes or eliminates the ability of the NS1 gene product to antagonize the cellular interferon response.[2]

Further, as of the effective filing date of the instant invention, the accepted age for embryonated eggs as a growth substrate for viral growth and propagation was 10-12 days, and not embryonated eggs less than 10 days old, as claimed in the instant invention. Immature embryonated eggs, such as six to seven day old eggs were *not* recognized by one of skill in the art as a substrate for viral growth and propagation, prior to the instant invention. In light of the fragile condition and small allantoic cavity of these young eggs, the prevailing view of those skilled in the art was that they were particularly unattractive for viral growth and propagation. Accordingly, there would have been no motivation for one of skill in the art, given the state of the art as of the effective filing date of the instant application to use an immature embryonated egg less than 10 days old for viral growth and propagation.

Applicants were the first to teach negative strand RNA viruses comprising mutations that result in an impaired interferon antagonist phenotype (*e.g.*, *see* the instant specification at page 14, line 1 to page 19, line 21). Specifically, Applicants were the first to teach methods for recombinantly engineering a negative strand RNA virus, in particular an influenza virus, so that the virus has an impaired interferon antagonist phenotype. For example, the instant invention for the first time sets forth attenuated influenza viruses with mutations, *e.g.*, truncations or deletions, in the NS1 gene that diminish or eliminate the ability of the NS1 gene product to antagonize the cellular interferon response (*see, e.g.*, the instant specification at page 14, line 23 to page 15, line 10; page 17, line 36 to page 18, line 12; Example 6, page 38, line 7 to page 42, line 30).

Additionally, Applicants were the first to teach that immature embryonated eggs less than 10 days old (*e.g.*, six day old embryonated eggs) provide a better growth substrate for attenuated negative strand RNA viruses with an impaired interferon antagonist phenotype, in particular recombinantly engineered attenuated negative strand RNA viruses with an

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[1] In Example 4, Mitsuhashi uses an Influenza virus A/Tokyo/6/73 strain, which is not a recombinant, attenuated virus containing a mutation in the NS1 gene.

[2] In the Abstract, Sasaki refers to the Hong Kong A influenza viruses, which are not a recombinant, attenuated virus containing a mutation in the NS1 gene.

impaired interferon antagonist phenotype, than older embryonated eggs (*e.g.*, 10 and 14 day old embryonated eggs) which are the conventional substrates for viral growth and production. The cited art does not recognize or appreciate that immature embryonated eggs less than ten days old are a better substrate for growth and propagation of negative strand RNA viruses with an impaired interferon antagonist pheonotype. In particular, Applicants demonstrated that an immature embryonated egg, especially its allantoic cavity, is an excellent growth substrate for attenuated influenza viruses having mutations in the NS1 gene that diminish or eliminate the ability of the NS1 gene product to antagonize the cellular interferon response. The cited art does not recognize or appreciate that immature embryonated eggs (*e.g.*, six day old eggs) are a better substrate for the propagation of influenza viruses with attenuated phenotypes, in particular attenuated phenotypes resulting from mutations in the NS1 gene of influenza virus that diminish or eliminate the ability of the NS1 gene product to induce a cellular interferon response. It was Applicants' unexpected discovery that negative strand RNA viruses with an impaired interferon antagonist phenotype grow to a higher titer in immature embryonated eggs that resulted in the recognition of immature embryonated eggs as a suitable substrate for the propagation of negative strand RNA viruses with an impaired interferon antagonist phenotype. Therefore, in view of the foregoing, compositions comprising immature embryonated eggs less than 10 days old containing a negative strand RNA virus with an impaired interferon antagonist activity are not rendered obvious over the references cited by the Examiner.

Applicants respectfully assert that the instant application sets forth at least one unexpected result of the claimed invention (*i.e.*, the ability of the viruses in the composition to grow to a higher titer in an immature embryonated egg as compared to an older egg). Applicants respectfully invite the Examiner's attention to Example 6 of the present application (*See* the instant specification at page 38, line 7 to page 42, line 31), whereby the unexpected result of the claimed compositions is unequivocally illustrated. In this working example, the ability of influenza viruses containing mutations in the NS1 gene which resulted in an impaired interferon antagonist phenotype to grow in embryonated chick eggs of different ages are compared to that of wild type influenza. As demonstrated in Table 3 (which is reproduced below for the Examiner's convenience), the differential growth properties of the influenza viruses containing mutations in the NS1 gene is much greater than that of wild type influenza, which was a *surprising and unexpected result.* As illustrated in Table 3, the difference in growth properties of an influenza virus containing a mutation in the NS1 gene between a 6-day old egg and a 10-day old egg is much more significant relative to that of wild type influenza. The wild type influenza virus reaches a 2-fold higher titer in a

10-day old egg vs. a 6-day old egg, whereas the influenza virus with a mutation in the NS1 gene reaches a 32 fold higher titer in a 10-day old egg vs. a 6-day old egg. Therefore, as demonstrated by the working example an attenuated negative strand RNA virus with an impaired interferon antagonist phenotype, such as an influenza virus with a mutation in the NS1 gene, grows unexpectedly better in an immature embryonated egg (*e.g.*, a 6-day old egg) vs. the conventional substrate used in the art (a 10-day old egg), relative to that of wild type virus.

TABLE 3: Virus Replication in Embryonated Chick Eggs

		HEMAGGLUTINATION TITERS[3]		
VIRUS	AGE OF EGGS	6 DAYS	10 DAYS	14 DAYS
WT PR8[4]		2,048	4,096	1,071
NS1/99		N.D.[5]	2,048	<2
DelNS1		64	<2	<2

Further, the efficacy of immature embryonated eggs, particularly 6-day old eggs for the propagation and growth of recombinantly engineered attenuated negative strand RNA viruses has been confirmed by post filing art. In particular, as evidenced by Mebatsion *et al.*, 2001 *Journal of Virology*, 75(1): 420-8 ("Mebatsion") which is submitted herewith as Exhibit C, New Castle Disease virus (NDV) mutants with a diminished interferon antagonist phenotype[6] (NDV-Vstop and NDV-6) do not propagate in 10-day old embryonated eggs, whereas they can grow to $2x10^2$ to $4x10^2$ focus forming units/mL in 6-day old embryonated eggs (*e.g.*, *See* Table 1 of Mebatsion which is reproduced below for the Examiner's convenience). Therefore, NDV mutants with a diminished interferon antagonist phenotype (mutants lacking part of the V protein which is known to be responsible for the interferon antagonist activity, as demonstrated in Park, Exhibit D) grow to a significantly higher titer in a 6-day old egg relative to a 10-day old egg, *e.g.*, 10^2 fold higher level. However, the wild type NDV virus grows to comparable titers both in young and older embryos (approximately 10^8 focus forming unit/mL). Therefore, these results confirm that immature embryonated eggs less than ten days old, *e.g.*, six days old, are a better substrate for propagation and growth of negative strand RNA viruses with an impaired interferon antagonist phenotype.

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[3] Titers represent the highest dilution with hemagglutination activity.
[4] Wild type influenza A/PR/8/34 virus.
[5] Not determined.
[6] See also Park *et al.*, 2003 *Journal of Virology*, 77(2): 1501-11; submitted herewith as Exhibit D, where the interferon antagonist phenotype of the V protein of NDV is established.

TABLE 1. INFECTIOUS TITERS AFTER PROPAGATION IN DIFFERENT SUBSTRATES

	INFECTIOUS TITERS[7] AFTER PROPAGATION IN			
VIRUS	BSR CELLS	VERO CELLS	6 DAY OLD EGGS	10 DAY OLD EGGS
NDV-6	$6X10^0$	$1X10^1$	$2X10^2$	0
NDV-Vstop	$6X10^0$	$1.4X10^2$	$4X10^2$	0
WILD TYPE	$4X10^3$	$7X10^5$	$1X10^8$	$1.2X10^8$

In view of Applicants' unexpected results which were confirmed by post-filing publications, examples of which are submitted herein, the claimed compositions are patentable even assuming, *arguendo*, a *prima facie* obviousness rejection were sound. *In re Chupp*, 816 F.2d 643 (Fed. Cir. 1987). Since the Applicants have demonstrated at least one unexpected benefit of the claimed compositions as compared to the cited art, the claimed compositions are not obvious over the art cited. In view of the foregoing, Applicants respectfully assert that the rejections under 35 U.S.C. § 103(a) cannot stand and should be withdrawn.

CONCLUSION

Applicants respectfully request entry and consideration of the foregoing remarks. Applicants believe that all of the present claims meet all of the requirements for patentability. Withdrawal of all rejections is requested.

If any issues remain, the Examiner is requested to telephone the undersigned at (212) 790-6431.

Respectfully submitted,

Date: June 11 , 2003

Laura A. Coruzzi 30,742
Laura A. Coruzzi (Reg. No.)

PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, New York 10036-2711
(212) 790-9090

By: Jennifer D. Chhoda
Reg No. 46,67

[7] Values are in focus-forming units per milliliter

EXHIBIT A

MARKED UP VERSION OF AMENDED CLAIMS

AS OF JUNE 11, 2003

IN U.S. PATENT APPLICATION NO. 09/724,419

ATTORNEY DOCKET NO. 6923-102-999

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43. The embryonated egg of claim 41 [43], wherein the embryonated egg is six days old.

50. The embryonated egg of Claim 42 [or 43] or 44, wherein the attenuated influenza virus is engineered to encode an epitope derived from another virus.

51. The embryonated egg of Claim 42 [or 43] or 44, wherein the attenuated influenza virus has a segmented genome comprising at least one segment derived from a different virus.

52. The embryonated egg of Claim [43] 44, wherein the attenuated influenza virus is genetically engineered.

53. The embryonated egg of Claim 42 [or 43] or 44, wherein the mutation in the NS1 gene is a deletion at the C-terminal of NS1.

55. The embryonated egg of Claim 42 [or 43] or 44, wherein the mutation in the NS1 gene is a deletion at the amino-terminal of NS1.

56. The embryonated egg of Claim 42 [or 43] or 44, wherein the influenza virus is influenza A or B virus.

57. The embryonated egg of Claim 42 [or 43] or 44, wherein the mutation in the NS1 gene is responsible for the attenuated phenotype of the influenza virus.

EXHIBIT B

PENDING CLAIMS

AS OF JUNE 11, 2003

IN U.S. PATENT APPLICATION NO. 09/724,419

ATTORNEY DOCKET NO. 6923-102-999

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41. An embryonated egg less than ten days old containing a recombinantly engineered attenuated negative strand RNA virus with impaired interferon antagonist activity, wherein said virus is not influenza C virus.

42. An embryonated egg less than ten days old containing a recombinantly engineered attenuated influenza virus having a mutation in the NS1 gene that diminishes or eliminates the ability of the NS1 gene product to antagonize the cellular interferon response, wherein said virus is not influenza C virus.

43. The embryonated egg of claim 41, wherein the embryonated egg is six days old.

44. An embryonated egg containing in the allantoic cavity an attenuated influenza virus having a mutation in the NS1 gene that diminishes or eliminates the ability of the NS1 gene product to antagonize the cellular interferon response, wherein the embryonated egg is six to nine days old and said virus is not influenza C virus.

45. An embyronated egg less than 10 days old containing delNS1.

46. The embryonated egg of Claim 41, wherein the negative strand RNA virus is influenza A virus, influenza B virus, respiratory syncytial virus, parainfluenza virus, mumps virus, measles virus, Newcastle disease virus, or vesicular stomatitis virus.

47. The embryonated egg of Claim 41 or 42, wherein the egg is a six to nine days old chick egg.

48. The embryonated egg of Claim 41, wherein the attenuated negative strand RNA virus is engineered to encode an epitope derived from another virus.

49. The embryonated egg of Claim 41, wherein the attenuated negative strand RNA virus has a segmented genome comprising at least one segment derived from a different virus.

50. The embryonated egg of Claim 42 or 44, wherein the attenuated influenza virus is engineered to encode an epitope derived from another virus.

51. The embryonated egg of Claim 42 or 44, wherein the attenuated influenza virus has a segmented genome comprising at least one segment derived from a different virus.

52. The embryonated egg of Claim 44, wherein the attenuated influenza virus is genetically engineered.

53. The embryonated egg of Claim 42 or 44, wherein the mutation in the NS1 gene is a deletion at the C-terminal of NS1.

54. The embryonated egg of Claim 53, wherein the NS1 gene encodes truncated NS1 proteins consisting of amino acid residues 1-60, amino acid residues 1-70, amino acid residues 1-90, amino acid residues 1-99, amino acid residues 1-100, amino acid residues 1-110, amino acid residues 1-120, amino acid residues 1-124, or amino acid residues 1-130 of the wild-type NS1.

55. The embryonated egg of Claim 42 or 44, wherein the mutation in the NS1 gene is a deletion at the amino-terminal of NS1.

56. The embryonated egg of Claim 42 or 44, wherein the influenza virus is influenza A or B virus.

57. The embryonated egg of Claim 42 or 44, wherein the mutation in the NS1 gene is responsible for the attenuated phenotype of the influenza virus.

71. The embryonated egg of Claim 41 or 42, wherein the egg is six to eight days old.

72. The embryonated egg of Claim 41 or 42, wherein the egg is six to seven days old.

73. The embryonated egg of Claim 41, wherein the attenuated negative strand RNA virus is engineered to encode a foreign antigen.

74. The embryonated egg of Claim 42 or 44, wherein the attenuated influenza virus is engineered to encode a foreign antigen.

75. The embryonated egg of Claim 44, wherein the egg is six to eight days old.

76. The embyronated egg of Claim 44, wherein the egg is six to seven days old.

77. The embryonated egg of Claim 41, wherein the attenuated negative strand RNA virus is engineered to encode a tumor antigen.

78. The embryonated egg of Claim 42 or 44, wherein the attenuated influenza virus is engineered to encode a tumor antigen.

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A Recombinant Newcastle Disease Virus with Low-Level V Protein Expression Is Immunogenic and Lacks Pathogenicity for Chicken Embryos

TESHOME MEBATSION,[1]* STEFAN VERSTEGEN,[1] LEONARDA T. C. DE VAAN,[1] ANGELA RÖMER-OBERDÖRFER,[2] AND CARLA C. SCHRIER[1]

Department of Virology, Intervet International B.V., 5830 AA Boxmeer, The Netherlands,[1] and Institute of Molecular Biology, Friedrich-Loeffler Institute, Federal Research Centre for Virus Diseases of Animals, D-17498 Insel Riems, Germany[2]

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Newcastle disease virus (NDV) edits its P-gene mRNA by inserting a nontemplated G residue(s) at a conserved editing site (3′-UUUUUCCC-template strand). In the wild-type virus, three amino-coterminal P-gene-derived proteins, P, V, and W, are produced at frequencies of approximately 68, 29, and 2%, respectively. By applying the reverse genetics technique, editing-defective mutants were generated in cell culture. Compared to the wild-type virus, mutants lacking either six nucleotides of the conserved editing site or the unique C-terminal part of the V protein produced as much as 5,000-fold fewer infectious progeny in vitro or 200,000-fold fewer in 6-day-old embryonated chicken eggs. In addition, both mutants were unable to propagate in 9- to 11-day-old embryonated specific-pathogen-free (SPF) chicken eggs. In contrast, a mutant (NDV-P1) with one nucleotide substitution (UUCUUCCC) grew in eggs, albeit with a 100-fold-lower infectious titer than the parent virus. The modification in the first two mutants described above led to complete abolition of V expression, whereas in NDV-P1 the editing frequency was reduced to less than 2%, and as a result, V was expressed at a 20-fold-lower level. NDV-P1 showed markedly attenuated pathogenicity for SPF chicken embryos, unlike currently available ND vaccine strains. These findings indicate that the V protein of NDV has a dual function, playing a direct role in virus replication as well as serving as a virulence factor. Administration of NDV-P1 to 18-day-old embryonated chicken eggs hardly affected hatchability. Hatched chickens developed high levels of NDV-specific antibodies and were fully protected against lethal challenge, demonstrating the potential use of editing-defective recombinant NDV as a safe embryo vaccine.

Newcastle disease virus (NDV) belongs to the genus *Rubulavirus* within the family *Paramyxoviridae*. Recent findings, however, have indicated that NDV is only distantly related to other members of the genus *Rubulavirus*, and it has been suggested that NDV should be assigned to a new genus within the subfamily *Paramyxovirinae* (6). NDV isolates are further categorized based on pathogenicity for chickens into velogenic, mesogenic, and lentogenic strains corresponding to high-, moderate-, and low-virulence strains, respectively. The molecular basis for this distinction lies mainly in the amino acid sequence of the protease cleavage site of the fusion (F) protein (14, 25). The precursor fusion glycoprotein F0 has to be cleaved into F1 and F2 for the progeny virus to be infectious and to be able to undergo multiple rounds of replication. Recently, experimental evidence for the presence of a direct correlation between the sequence of the cleavage site and NDV virulence was provided by changing the protease cleavage site of a lentogenic strain of NDV (GGRQGR↔ L) into the consensus cleavage site of a velogenic strain (GRRQRR↔ F). A dramatic increase in virulence of the genetically modified virus indicated that the key determinant for NDV virulence is the cleavage efficiency of the precursor protein (28). However, there is indirect evidence suggesting that cleavage efficiency is not the sole determinant governing NDV virulence (22, 28).

The negative-strand RNA virus genome of NDV contains six genes encoding six major structural proteins (3′-NP-P-M-F-HN-L-5′). A general feature of the *Paramyxovirinae*, however, is the presence of additional structural or nonstructural viral proteins resulting from the use of alternative reading frames and RNA editing of their P genes (19). Like other members of the *Paramyxovirinae*, NDV edits its P gene by inserting one or two G residues at the conserved editing locus (UUUUUCCC) and transcribes three P-gene-derived mRNA species. The mRNAs encode the open reading frame (ORF) of P (unedited), the V ORF (with a +1 frameshift), and the W ORF (with a +2 frameshift) (39). These proteins are amino coterminal and vary at their carboxy-terminal ends in length and amino acid composition. Of the three P-gene products, the P protein is known to be an essential component for viral RNA synthesis and, together with the L protein, was demonstrated to form an active transcriptive complex (15). However, not much is known about the two other P-gene products. The V protein is of particular interest since it is conserved in all three genera of the *Paramyxovirinae*, with the exception of human parainfluenza virus type 1 (HPIV-1), which lacks an intact V ORF (23). Moreover, the V protein is characterized by the presence of a highly conserved cysteine-rich carboxy-terminal domain, and there is evidence that this domain of simian virus (SV5) interacts with damage-specific DNA binding protein (21). The V

* Corresponding author. Mailing address: Department of Virology, Intervet International B.V., P.O. Box 31, 5830 AA Boxmeer, The Netherlands. Phone: 31 485 587 351. Fax: 31 485 587 339. E-mail: teshome.mebatsion@intervet.com.

proteins of NDV and SV5 were shown to bind zinc and were also demonstrated to be structural components of virions (26, 33, 38). On the other hand, the V proteins of Sendai virus (SeV) and measles virus (MV) are not structural components of virions and are not associated with the ribonucleoprotein complex (16, 20).

Further insight into the functions of the additional P-gene products of the *Paramyxoviridae* was obtained after the development of reverse genetics technology, which enabled genetic manipulation of the genomes of nonsegmented negative-strand RNA viruses (reviewed in references 5 and 31). Studies with SeV and MV showed that the V and/or W protein could be deleted without detrimental effects on replication of the virus in cell culture (7, 8, 17, 18, 35). Interestingly, however, the editing-defective SeV was found to replicate normally in vitro but was severely attenuated in pathogenicity for mice (8, 17, 18). The mechanism of the in vivo attenuation in certain members of the *Paramyxoviridae* may involve the interferon (IFN) system, in which accessory proteins, particularly V or C proteins (20), are responsible for blocking the activation of IFN-responsive genes (9, 10, 13).

NDV is responsible for one of the most devastating diseases of poultry and has substantial economic impact in the poultry industry. Vaccination of chickens, particularly those raised for commercial consumption, is carried out throughout the world. The currently available live attenuated ND vaccines can be administered to hatched chickens only in drinking water, aerosols, or eye drops or by parenteral routes. These methods of applications have several disadvantages, the most important being labor costs. Embryo, or in ovo, vaccination has proved to be an effective and economical method of application for several commonly used vaccines, such as those for turkey herpesvirus and infectious bursal disease virus (36, 37). Moreover, in ovo vaccination was found to be advantageous due to the administration of a uniform dose of vaccine into each egg using automated machines. However, several live virus vaccines for poultry cannot be administered in ovo mainly because they cause high embryo mortality. For NDV, the use of a modified live vaccine for in ovo administration has been described previously (1). However, this involves the use of a chemical mutagenic agent, ethyl methanesulfonate, at each step of the vaccine preparation. Recombinant fowlpox vectors expressing NDV fusion protein and/or hemagglutinin-neuraminidase protein have been successfully constructed, and their safety and efficacy for in ovo vaccination have been studied in specific-pathogen-free (SPF) chickens (12). Although the recombinant vaccines were shown to be efficacious in SPF animals, no data were provided on the efficacy of such recombinant vaccines in commercial chickens with neutralizing maternal antibodies. Such passive antibodies, which are usually present at high levels in very young chickens from immunized parent flocks, can impair the effectiveness of live virus vaccines. Since conventional live ND vaccines confer full protection even in the presence of maternal antibodies, it is highly desirable that the currently available posthatching vaccines be further attenuated to make them suitable for embryo vaccination.

Recently, the recovery of infectious lentogenic NDV from full-length cDNA has been described (28, 32). We demonstrated that the recombinant virus was phenotypically identical to its parent virus, NDV Clone-30, which is currently used as a live posthatching vaccine (32). In the present study, this recombinant cDNA technology was used to introduce mutations into the conserved editing site of the P gene. A single U-to-C change within the U stretch substantially reduced the editing frequency and hence considerably lowered the level of additional proteins generated by RNA editing. The editing-defective virus was dramatically attenuated for chicken embryos. Here, we describe the effects of this and other mutations on viral replication and pathogenesis and discuss the potential use of such editing-defective viruses for the development of ND vaccines that can be used to immunize chicken embryos.

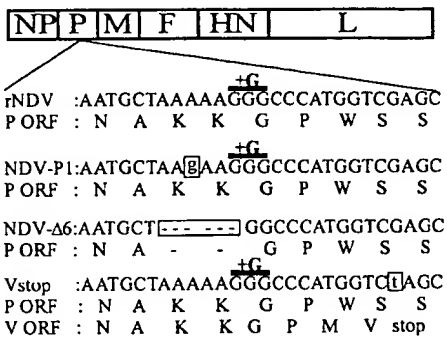

FIG. 1. Recombinant NDV constructs. A schematic representation of the NDV gene order in the negative-strand genomic RNA is shown. Sequences around the editing site (positions 2274 to 2300) are presented in a positive sense. The modifications resulting in interruption of the A stretch in NDV-P1, deletions of six nucleotides of the conserved editing site in NDV-Δ6, and the creation of a stop codon in the *trans*-V frame of NDV-Vstop (Vstop) are shown in boxes. +G indicates the position for insertion of nontemplated G residue(s).

MATERIALS AND METHODS

Viruses and cells. A recombinant NDV, rNDV, which was generated from a full-length cDNA copy of the lentogenic ND vaccine virus Clone-30 was described previously (32). A lentogenic posthatching ND vaccine, NDW, was obtained from a commercial source (Fort Dodge). The velogenic Herts strain 33/56 of NDV was used for challenge purposes. BSR-T7/5 cells stably expressing phage T7 RNA polymerase (4) were used to recover infectious NDV from cDNA.

Introduction of mutation into the full-length NDV cDNA. The plasmid pflNDV, expressing the full-length antigenome RNA of Clone-30 (32), was used to introduce mutations. Since NDV edits its P-gene mRNA by inserting nontemplated G residues (39), we modified the conserved editing site (UUUUUCCC) in the P gene of pflNDV. PCR was performed with the template pflNDV using forward primer 4 (5′-GCTCCTCGCGGCTCAGACTCG-3′, nucleotides 151 to 171) and reverse primers 1 (5′-CCATGGGCCCTTCTTAGCATTGGACG-3′, nucleotides 2269 to 2294) and 3 (5′-CCATGGGCCCGCATTGGACG-3′, nucleotides 2269 to 2294) to introduce one nucleotide change and a deletion of six nucleotides, respectively (Fig. 1). PCR products were then digested with *Aat*II and *Apa*I and cloned into the same sites of pflNDV. To selectively block expression of the unique C-terminal part of the V protein, a stop codon was introduced into the *trans*-V frame without affecting the P frame. PCR was performed using primer 20 (5′-CCCGGGAATCTTCTCTGGCGC-3′, nucleotides 3764 to 3784) and primer 29 (5′-AAGGGCCCATGGTCTAGCCCCCAAGAG-3′, nucleotides 2283 to 2309). The product was digested with *Apa*I and *Rsr*II and ligated into the same site of pflNDV. The nucleotide numbering is based on that of Römer-Oberdörfer et al. (32). The region newly introduced into each clone was sequenced to rule out PCR-introduced errors. The resultant full-length clones, with one nucleotide substitution at the editing site, a deletion of six nucleotides, or the insertion of a stop codon in the V ORF, were named NDV-P1, NDV-Δ6, and NDV-Vstop, respectively (Fig. 1).

In order to be able to grow and characterize the mutants in vitro without the addition of proteolytic enzymes, additional mutations were introduced at the F protein cleavage site. First, a 3.3-kb *Apa*I-*Acl*I fragment of pflNDV was cloned

into the *Sma*I site of the pUC18 vector. F protein cleavage site modification was performed using a site-directed mutagenesis kit (Amersham Pharmacia Biotech) with primer MP1 (5'-CTGTGACTACATCTGGAGGGCGGAGACAGAAGC GCTTTATAGGCGCCATT ATTGG-3', nucleotides 4857 to 4911) according to the supplier's instructions. The modified plasmid was then digested using *Pml*I and *Not*I, and a fragment of approximately 1.2 kb was used to replace the corresponding fragment of pflNDV. The resultant full-length clone was then digested with *Pml*I and *Bsi*WI, and a fragment of approximately 5.1 kb containing the modified F cleavage site was used to replace the corresponding fragments of NDV-Δ6 and NDV-Vstop.

Recovery of recombinant viruses. Approximately 1.5×10^6 BSR-T7/5 cells stably expressing phage T7 RNA polymerase (4) were grown overnight in 3.2-cm-diameter dishes. Cells were transfected with plasmid mixtures containing 5 μg of pCite-NP, 2.5 μg of pCite-P, 2.5 μg of pCite-L, and 10 μg of one of the full-length clones using a mammalian transfection kit ($CaPO_4$ transfection protocol; Stratagene). Three to five days after transfection, supernatant was harvested and inoculated into the allantoic cavities of 9- to 11-day-old embryonated SPF chicken eggs. After 3 to 4 days of incubation, the presence of virus in the allantoic fluid was determined by a rapid plate hemagglutination (HA) test using chicken erythrocytes (3). Supernatants obtained from transfections involving full-length clones with modifications at the F cleavage site were serially passaged in BSR cells. Virus stocks were prepared from supernatants of infected BSR cells, and the infectious titers were determined by serial 10-fold dilutions and staining of infectious foci with an anti-F monoclonal antibody (MAb). The growth characteristics of the viruses were then analyzed in BSR and Vero cells as well as in 6- and 10-day-old embryonated SPF chicken eggs.

Reverse transcription-PCR and determination of P-gene mRNA editing frequency. BSR-T7/5 cells were infected with the recombinant viruses, and total RNA was prepared 24 to 36 h after infection using the RNeasy kit (Qiagen). Reverse transcription by avian myeloblastosis virus reverse transcriptase on 1 μg of total RNA was primed with NDV P-gene-specific oligonucleotide P13 (5'-C CACCCAGGCCACAGACGAAG-3', nucleotides 2176 to 2196) or oligo(dT) primer to amplify only mRNAs. DNA amplification was then performed with primers P13 and P17 (5'-ATGAATTCAGCTGTTGGA-3', nucleotides 2680 to 2696). The PCR products were analyzed on a 1% agarose gel and used directly for sequencing or were digested with *Eco*RV and *Sal*I and ligated into the same site of the pSKT7T vector. Cloned plasmids were sequenced from independent colonies and examined for the presence or absence of insertion of a nontemplated G residue(s) at the editing site.

Immunofluorescence analysis. For the analysis of viral protein expression, BSR-T7/5 cells were infected with the recombinant viruses and incubated for approximately 18 h. Infected cells were fixed for 1 h at room temperature with cold ethanol (96%). Cells were then incubated with antipeptide rabbit serum directed against the 16 C-terminal amino acids of V protein or MAbs reacting with NP or F protein. Cells were washed and stained with fluorescein isothiocyanate-conjugated anti-rabbit or anti-mouse antibody containing 0.05% Evans blue and examined by fluorescence microscopy.

Immunoblotting. For virus purification, 9- to 11-day-old embryonated SPF chicken eggs were infected and allantoic fluid was collected 3 to 4 days postinfection. Virus in the allantoic fluid was then purified and concentrated by centrifugation through a 20% sucrose cushion in a Beckman SW28 rotor at 21,000 rpm for 90 min. The pellet was resuspended and mixed with protein sample buffer to disrupt the virions. Viral proteins from purified virions were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore), and incubated with antipeptide serum specific for the C-terminal 16 amino acids of the V protein of NDV Clone-30 or with a MAb specific for NDV NP protein. Membranes were then incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G. Proteins were visualized after incubation with peroxidase substrate (Vector).

Virus propagation in embryonated eggs. To determine virus titers and embryo mortality, serial 10-fold dilutions of the recombinant viruses were prepared, and 9- to 11-day-old embryonated SPF chicken eggs were inoculated in the allantoic cavity with the serial dilutions, in duplicate. A rapid-plate HA test (3) was carried out on one set of eggs after 4 days of incubation, and the titer, expressed as the 50% embryo-infectious dose (EID_{50}), was calculated using the method of Reed and Muench (29). The remaining eggs were observed daily for embryo mortality for at least 7 days, and the 50% embryo-lethal dose was then determined using the same method. To determine the susceptibility of chicken embryos to NDV-P1 infection at an early age of embryonation, chicken embryos at the ages of 7 and 8 days were infected with 2 $\log_{10}$ EID_{50} and observed for 1 week.

In ovo vaccination and challenge. Eighteen-day-old embryonated SPF or commercial chicken eggs were inoculated through a hole punched at the blunt end of the egg. Using a 23-gauge needle, 0.1 ml of the virus dilution or negative allantoic fluid was injected just below the air membrane. Eggs were further incubated until hatching. The percent hatchability was recorded, and chickens were observed daily for general health. At 14 days of age, chickens were weighed and blood samples were taken. Serum samples were assayed for NDV antibodies in the NDV hemagglutination inhibition test (3). At 14 days of age (~17 days postvaccination), all animals were challenged with intramuscularly administered virulent Herts strain of NDV. Chickens were observed daily for a period of 10 days for clinical signs of disease or mortality.

RESULTS

Generation of mutant NDV from cDNA. In order to disrupt the conserved P-gene mRNA editing or selectively block expression of the unique C-terminal part of the V protein, the modifications shown in Fig. 1 were carried out on the full-length cDNA clone (pflNDV) of NDV Clone-30 (32). Each modified full-length cDNA clone, together with three support plasmids expressing NDV NP, P, and L proteins, was transfected into BSR-T7/5 cells. Transfection experiments were also performed with the unmodified full-length cDNA, pflNDV, to compare rescue efficiencies. After 3 to 5 days of incubation, supernatants were harvested and transfected cells were subjected to immunofluorescence staining using an anti-F MAb. At least 20 to 50 immunofluorescence-positive cells were detected in all of the transfection experiments involving pflNDV or modified full-length clones, showing that there were genome replication and expression of viral proteins in cell culture.

Embryonated SPF chicken eggs, which have long been known as the best substrates for propagation of lentogenic NDVs (14, 25), were then inoculated with transfection supernatants. After 3 to 4 days of incubation, allantoic fluid samples were harvested and subjected to an HA test. HA was detected in eggs inoculated with the supernatant from cells transfected with the pflNDV. However, two extra egg passages were required for NDV-P1 to be detected using the HA test, suggesting that this mutant grows slowly when inoculated into the allantoic cavity of 9- to 11-day-old embryonated SPF chicken eggs. Surprisingly, infectious virus was not detected in the allantoic fluid of embryonated eggs inoculated with supernatants obtained from NDV-Vstop and NDV-Δ6 transfections, even after four successive passages. In spite of three repeated rescue experiments, we were unable to detect infectious virus in the allantoic fluid after passage in 9- to 11-day-old embryonated eggs.

V protein of NDV is essential for efficient virus propagation. In order to determine whether these mutants grow in cell culture as efficiently as the wild-type virus, repeated passage in culture cells is necessary. However, due to the absence of efficient cleavage of the precursor F protein, lentogenic NDVs cannot be propagated in most tissue culture systems without the addition of proteases. In contrast, velogenic strains are able to undergo multiple rounds of replication in cell culture. To be able to grow the mutants in vitro, "virulent" versions of rNDV, NDV-Vstop, and NDV-Δ6 were constructed by modifying the F cleavage site. The alterations resulted in a change of the F cleavage site of Clone-30 (GGRQGR↔ L) to a cleavage site similar to that of a virulent strain (GRRQKR↔ F). Using an anti-V-peptide serum specific for the C terminus of V protein, the absence of V expression in both mutants was confirmed, demonstrating that the introduced mutations were sufficient to completely abolish RNA editing. In order to compare the

TABLE 1. In vitro and in vivo propagation of V-deficient NDV mutants that posses an F protein cleavage site similar to that of a virulent NDV strain

Virus	Infectious titers[a] after propagation in:			
	BSR cells	Vero cells	6-day-old embryos	10-day-old embryos
NDV-Δ6	6×10^{9}	1×10^{1}	2×10^{2}	0
NDV-Vstop	6×10^{9}	1.4×10^{2}	4×10^{2}	0
Wild type	4×10^{3}	7×10^{5}	1×10^{8}	1.2×10^{8}

[a] For in vitro propagation, BSR and Vero cells were infected at a multiplicity of infection of 0.001, and the infectious titers in supernatants harvested after 4 days of infection were determined using end point dilutions in cell culture. For in ovo propagation SPF chicken eggs at 6 or 10 days of embryonation were inoculated with 1.7 $\log_{10}$ focus-forming units/egg and incubated for 4 days. Infectious titers in the allantoic fluid were then determined in cell culture after endpoint dilutions. Values are in focus-forming units per milliliter.

growth efficiency of the mutants with that of the wild-type virus, BSR and Vero cells were inoculated with the respective supernatants at a multiplicity of infection of 0.001. In addition, 6- and 10-day-old embryonated SPF chicken eggs were inoculated with 1.7 $\log_{10}$ focus-forming units/egg. Infected cell cultures and embryonated eggs were incubated for 4 days, and the titers of infectious viruses in cell culture supernatants or allantoic fluid were determined. In vitro, the titers of both mutants were 600- to 5,000-fold lower than the titers of the wild-type virus depending on the type of cells (Table 1). This remarkable growth impairment of both mutants in cell culture indicates that V protein plays a crucial role in NDV replication. In 6-day-old chicken embryos, both mutants yielded more than 200,000-fold-lower titers than the wild-type virus (Table 1) and did not cause any embryo mortality. Interestingly, the mutants were completely unable to propagate in 10-day-old embryonated eggs, even after serial passage, indicating that V is also a pathogenesis factor. The wild-type virus grew to identical titers in younger and older embryos and caused mortality of up to 100%.

NDV-P1 expresses a low level of V protein. The mutant which could be propagated in embryonated eggs, NDV-P1, was then serially passed two or three times in 9- to 11-day-old embryonated eggs. The infectious titers of this mutant after the fifth and sixth egg passages were 6.7 and 7.1 $\log_{10}$ EID_{50} per ml, respectively, which were at least 100-fold lower than the titer obtained for the parent virus after the third egg passage (9.2 $\log_{10}$ EID_{50} per ml). Experiments described here were carried out using the sixth passage of NDV-P1 except where it is stated that the fifth passage was employed. BSR-T7/5 cells were infected with NDV-P1 or the parent virus rNDV and subjected to immunofluorescence analysis. Using MAbs directed against NDV NP or F protein, the levels and patterns of NP and F protein fluorescence in cells infected with the mutant and the parent virus were indistinguishable (Fig. 2). In contrast, an anti-V peptide serum specific for the C terminus of V protein reacted with intense fluorescence only with cells infected with the rNDV. The same dilution of the serum revealed a specific but very weak fluorescence in NDV-P1-infected cells, suggesting a low level of V expression (Fig. 2).

V protein is a structural component of NDV; therefore, it was of interest to determine whether the low level of V expression in infected cells would lead to low-level incorporation of V into virions. Thus, virions purified and concentrated through 20% sucrose were subjected to immunoblotting experiments. Using an NP-specific MAb, which is reactive with the NP protein of both viruses with equal sensitivity, it was possible to standardize the amount of protein loaded into the gel (Fig. 3). Although comparable amounts of rNDV and NDV-P1 proteins were subjected to the Western blot analysis, the amount of V protein of NDV-P1 was considerably smaller than that of rNDV, demonstrating low-level V protein incorporation into NDV-P1 virions. Analysis of diluted samples by Western blotting revealed that the V protein content of NDV-P1 virions was approximately 20-fold lower than that of rNDV.

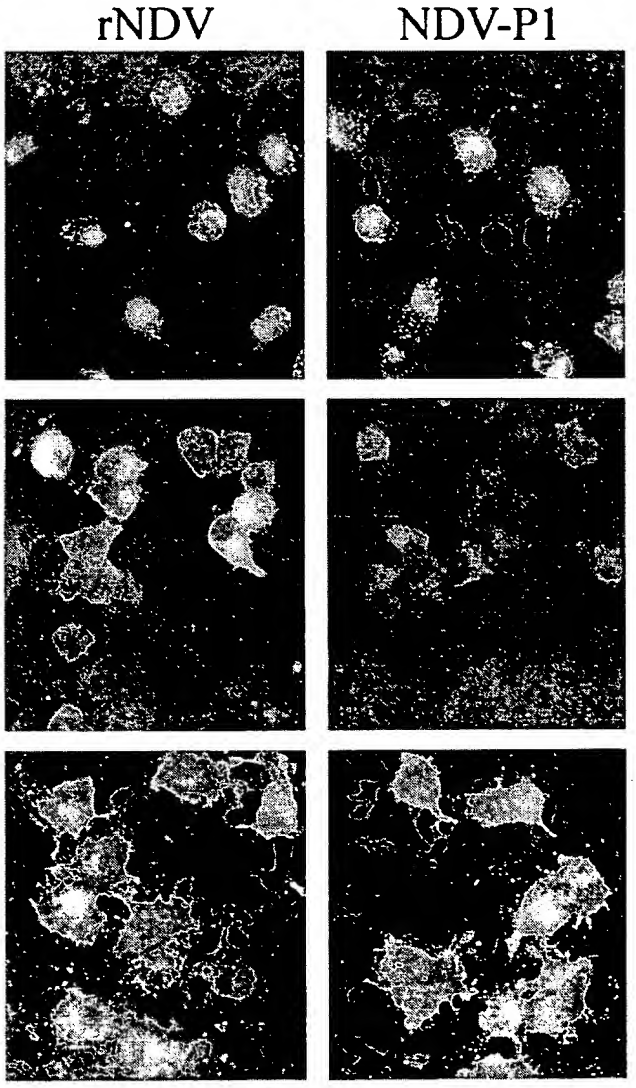

FIG. 2. Low-level V protein expression in NDV-P1-infected cells. BSR-T7/5 cells were infected with rNDV or NDV-P1 at a multiplicity of infection of approximately 0.01. Eighteen hours after infection, cells were processed for indirect immunofluorescence after incubation with MAbs specific for NP protein (top) or for F protein (bottom) or anti-V peptide serum (middle). Although the levels of NP and F protein expressions in cells infected with both viruses were indistinguishable, the level of V protein expression was considerably lower in cells infected with NDV-P1 than in those infected with rNDV.

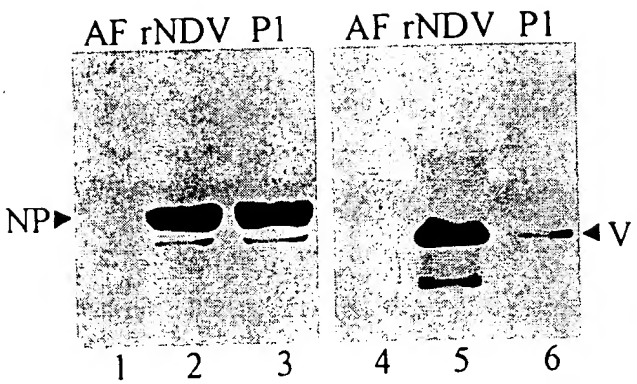

FIG. 3. NP and V proteins of sucrose-purified recombinant viruses. Virions in the allantoic fluid of infected embryonated eggs were purified by centrifugation through 20% sucrose. The volumes loaded for NDV-P1 were 4.5-fold greater than those for rNDV in order to normalize for NP protein content. Samples were loaded in duplicate, and blots were incubated with anti-NP MAb (lanes 1 through 3) or with anti-V peptide serum (lanes 4 through 6). AF, allantoic fluid from noninfected embryonated eggs; P1, NDV-P1.

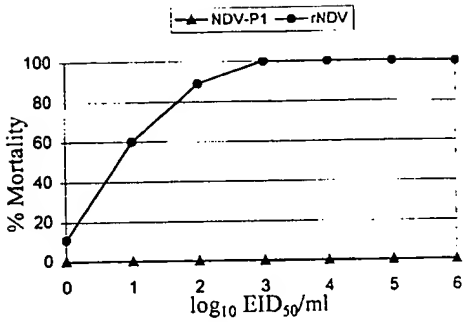

FIG. 5. Pathogenicity of rNDV and NDV-P1 in SPF chicken embryos. Eleven-day-old embryonated SPF chicken eggs were inoculated with the parent rNDV (passage 3) or the mutant NDV-P1 (passage 5) and incubated for 7 days or until the embryos had died. NDV-P1 caused no embryo mortality for 7 days at all indicated doses (0.2 ml/egg), whereas rNDV was lethal at a dose as low as 1 EID_{50}/ml (approximately 10% mortality). Embryos inoculated with rNDV started to die as early as 3 days postinoculation at higher doses.

As the V protein of NDV can be produced only by the RNA editing process, we determined the sequence around the editing locus from a total of 72 independent colonies of plasmids derived from NDV-P1. As expected, we found a plasmid containing an insertion of one nontemplated G residue leading to V-ORF (1.4%), in spite of the presence of a modification at the editing site (Fig. 4). For comparison, 41 independent colonies were sequenced for rNDV; 28 out of 41 (68.3%) of the sequenced plasmids encoded the unedited version of P protein, and 12 out of 41 (29.3%) encoded the V protein with an insertion of one nontemplated G residue. Only one plasmid out of 41 (2.4%) possessed an insertion of two nontemplated G residues and hence encoded W protein. The frequency of RNA editing of the wild-type virus is very similar to the results obtained by Steward et al. (39), except that plasmids encoding W protein were approximately threefold less abundant in this study. Compared with the wild-type virus, the NDV-P1 virus edits its P-gene mRNA at an approximately 20-fold lower frequency and hence synthesizes V protein at a correspondingly low level. Taken together, these results showed that the substitution that interrupts the U stretch at the editing locus did not completely block P-gene mRNA editing but dramatically reduced the RNA editing frequency.

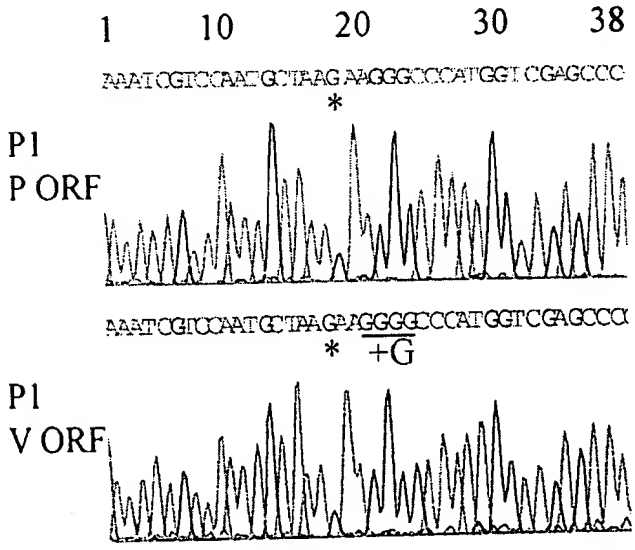

FIG. 4. P-gene mRNA editing in NDV-P1-infected cells. mRNA sequences in the regions of the editing site with the unedited P ORF or with insertion of one G residue (+G) coding for V ORF are shown. NDV-P1 (P1) edits its P-gene mRNA in spite of the interruption of the five A residues by A-to-G substitution (*).

NDV-P1 is attenuated for chicken embryos. NDV isolates vary in their virulence for chicken embryos as well as for chickens. The degree of virulence of a given NDV isolate can be measured by assessing the pathogenicity of the virus for 1-day-old chickens after intracerebral inoculation (2). Using this method, the intracerebral pathogenicity index of the rNDV was found to be identical to that of the wild-type parent, Clone-30 (32). Another method is to evaluate the time required for the virus to cause embryo mortality after allantoic inoculation. To determine the embryo mortality caused by NDV-P1, 10-fold serial dilutions of passage 5 of NDV-P1 were inoculated into 11-day-old embryonated SPF chicken eggs (0.2 ml/egg), which were then incubated for 1 week. Interestingly, no specific embryo mortality was detected during the 7-day incubation period, showing that NDV-P1 was not lethal for embryos even with a dose as high as 6 $\log_{10}$ EID_{50}/ml (Fig. 5). Chicken embryos inoculated with the parent virus, rNDV, started to die as early as 3 days postinoculation, at doses higher than 4 $\log_{10}$ EID_{50}/ml. The difference between the 50% infectious dose and 50% lethal dose of rNDV was only 0.3 $\log_{10}$. In contrast, this difference was as high as 6.7 $\log_{10}$ for NDV-P1, showing that it was attenuated at least 10^6-fold more than its parent virus. To further analyze the pathogenicity of NDV-P1 for younger embryos, 7- and 8-day-old embryonated SPF chicken eggs were inoculated at a dose of 2 $\log_{10}$ EID_{50}/egg and observed for 1 week for embryo mortality. Interestingly, NDV-P1 was capable of causing embryo mortality reaching 62 and 23% for 7- and 8-day-old embryos, respectively. NDV-P1

TABLE 2. Hatchability and body weight of chickens after in ovo vaccination

Virus	Dose[a]	Hatchability (%)[b]	Wt[c]
NDV-P1	3.5	22 (73)	133
NDV-P1	4.3	28 (93)	135
NDV-P1	5.4	21 (70)	115
rNDV	5.0	7 (23)	ND
NDW	5.1	7 (23)	85
Control	0	29 (96)	141

[a] Log_{10} EID_{50} per egg calculated after back titration of the samples.
[b] Number of chickens hatched from 30 eggs.
[c] Mean body weight in grams at 2 weeks of age. ND, not determined.

reached a 10-fold-higher titer in these younger embryos than the virus grown in 9- to 11-day-old embryonated eggs. NDV-P1 was not lethal for SPF chicken embryos after 8 days of embryonation, indicating an age-dependent resistance of chickens to disease caused by NDV-P1.

In ovo vaccination of SPF chicken embryos with NDV-P1. NDV-P1 did not cause embryo mortality when applied to 9- to 11-day-old embryos; therefore, an in ovo (embryo) vaccination experiment was carried out to determine the safety of NDV-P1 in older embryos. We chose to perform this experiment in 18-day-old embryonated SPF chicken eggs because commercially available embryo vaccines are routinely administered at this age of embryonation. Hatchability was found to be up to 93% for NDV-P1-vaccinated chickens, compared to 96% for the control group (Table 2). The lowest hatchability (23%) was seen in eggs inoculated with either the parent rNDV or NDW, a live attenuated posthatching vaccine. At 2 weeks of age, the mean body weights of chickens hatched from NDV-P1-inoculated eggs ranged from 115 to 135 g, compared to 85 g for the animals that had received a comparable dose of NDW (Table 2).

NDV-P1 protects SPF chickens against a lethal challenge. To determine whether protective antibodies were induced in chickens hatched from eggs vaccinated in ovo, blood samples were collected at 2 weeks of age and animals were challenged with a velogenic Herts strain of NDV. Chickens vaccinated as embryos with NDV-P1 developed high antibody levels in a dose-dependent manner (Table 3). Interestingly, the level of protection against lethal challenge reached more than 95% in a dose-dependent manner. All control chickens died within 3 days of challenge. These data show that NDV-P1 can confer full protection when administered to 18-day-old SPF chicken embryos.

TABLE 3. NDV-P1 applied at day 18 of embryonation protects SPF chickens against lethal NDV challenge

Virus	Dose (log_{10} EID_{50}/egg)	Mean HI[a]	Survival (%)[b]
NDV-P1	3.5	4.0 ± 1.1	19/20 (95)
NDV-P1	4.3	4.8 ± 1.0	20/20 (100)
NDV-P1	5.4	5.4 ± 1.2	19/19 (100)
Control	0	0.7 ± 0.5	0/20 (0)

[a] HI, log_2 hemagglutination-inhibition titer at 2 weeks of age.
[b] Chickens were challenged with the Herts strain of NDV (5.5 log_{10} EID_{50}/chicken) intramuscularly.

TABLE 4. Safety and efficacy of NDV-P1 in commercial chickens vaccinated in ovo at 18 days of embryonation

Virus	Dose[a]	Hatchability (%)[b]	HI[c]	Body wt[d]	Survival (%)[e]
NDV-P1	3.7	29 (96)	1.4 ± 1.0	439	7/20 (35)
NDV-P1	4.5	29 (96)	1.5 ± 0.9	413	15/20 (75)
NDV-P1	5.7	27 (90)	1.8 ± 1.1	438	17/20 (85)
Control		29 (96)	1.2 ± 0.9	440	4/20 (20)

[a] Log_{10} EID_{50} per egg, calculated after back titration of the samples.
[b] Number of chickens hatched from 30 eggs.
[c] HI, hemagglutination-inhibition titer (log_2) against NDV at 2 weeks of age.
[d] Mean body weight in grams at 2 weeks of age.
[e] Chickens were challenged with the Herts strain of NDV at a dose of 5.5 log_{10} ELD_{50}/chicken intramuscularly.

NDV-P1 in commercial chicken embryos. In the study involving SPF chickens in which NDV specific antibodies are absent, a dose as low as 3.5 log_{10} EID_{50} protected 95% of the animals. In contrast, embryos from commercial chickens acquire passive immunity by the transfer of maternal immunoglobulins from serum to egg yolk. Such passive antibodies, which can be present at high levels (4 to 7 log_2 hemagglutination inhibition units) in very young chickens from immunized parent flocks, might impair the effectiveness of live virus vaccines by neutralizing the vaccine virus. To examine the safety of NDV-P1 and its ability to confer protection in the presence of maternally derived antibody, in ovo vaccination of commercial chicken embryos was performed. Hatchability of embryonated commercial chicken eggs was not affected by in ovo administration of NDV-P1 (Table 4). Moreover, the body weights of all groups of chickens vaccinated with NDV-P1 were comparable to those of the unvaccinated control group, demonstrating the safety of NDV-P1 when administered in ovo to 18-day-old embryonated commercial chicken eggs. The level of antibody response and protection of chickens vaccinated as embryos with NDV-P1 depended on the dose administered (Table 4). In the group that had received the highest dose, 85% of the chickens were protected against challenge, demonstrating the ability of NDV-P1 to break through maternal antibody and confer protection.

DISCUSSION

The V protein of the *Paramyxoviridae* is one of the most conserved P-gene-derived accessory proteins and is characterized by a cysteine-rich C-terminal region. Based on in vitro and in vivo results, the V protein and other P-gene-derived accessory proteins of members of the *Paramyxoviridae* were categorized as nonessential gene products. In this study, NDV mutants lacking V protein showed severe growth impairment in vitro and in 6-day-old embryonated chicken eggs. In contrast, no virus growth could be detected in 9- to 11-day-old embryonated eggs, indicating that V protein plays a dual role in virus replication and pathogenesis. Apart from the mutants completely lacking V protein, we succeeded in recovering attenuated NDV by introducing specific mutations at the conserved editing locus, which resulted in down regulation of V protein expression instead of complete abrogation.

It has long been documented that lentogenic NDVs are unable to produce infectious viruses in most tissue culture

systems without the addition of proteases. This is mainly due to the absence of efficient cleavage of the precursor fusion protein F0 to FI and F2 (25). Chicken embryos, in contrast to cell cultures, support the propagation of lentogenic NDVs to high titers and are obviously the best choices for the propagation of newly generated recombinant viruses. Thus, transfection supernatants were passed into 9- to 11-day-old embryonated SPF chicken eggs. However, apart from the rNDV, the only viable recombinant virus that was recovered after passage in embryonated eggs was NDV-P1. The mutant NDV-P1, in spite of the one nucleotide substitution at the editing site, was found to edit its P-gene mRNA, albeit at a 20-fold-lower frequency. NDV-P1 was able to propagate autonomously in 9- to 11-day-old embryonated eggs and reached a peak titer of 7.1 $\log_{10}$ EID_{50}/ml after six egg passages. Interestingly, a 10-fold-higher titer was obtained when this mutant was grown in 7- or 8-day-old embryos. In contrast, NDV-Vstop and NDV-Δ6 mutants were unable to grow in 9- to 11-day-old embryonated eggs despite repeated rescue and passage experiments. In order to be able to propagate the mutants in cell culture, we constructed virulent versions of the mutants and the wild-type virus by modifying the F protein cleavage site. Compared with the virulent wild-type virus, the mutant viruses required one or two extra cell culture passages to produce cytopathic effects in approximately 80% of infected BSR cells, suggesting that abolition of V expression may lead to prolonged replication. The mutant viruses showed severe impairment in replication in both BSR and Vero cells and grew to titers which were as much as 5,000-fold lower than the titer of the virulent wild-type virus, demonstrating the requirement of V protein for efficient virus replication in vitro. The difference in growth between the wild type and the mutants was very dramatic in embryonated eggs. Although the virulent wild-type virus grew to identical titers both in young and older embryos (approximately 10^8 focus-forming units/ml), the mutants grew to more than 200,000-fold-lower titers in 6-day-old embryonated eggs. In 9- to 11-day-old embryonated eggs, which are commonly used for NDV propagation, no virus growth could be detected. This indicates that V plays an important role in NDV pathogenesis in addition to its involvement in virus replication.

The mutant NDV-Vstop was constructed in order to distinguish the role played by V from that of W. The severely impaired in vitro and in vivo growth of NDV-Vstop, therefore, provides evidence that the cysteine-rich C terminus of V protein was mainly responsible for this incompetence. A mutant of SeV lacking the cysteine-rich C-terminal portion of V protein was also attenuated in vivo but replicated well in vitro, suggesting that this portion of the V protein is particularly responsible for in vivo attenuation (18). However, our results demonstrate that V protein is not only a pathogenesis factor in vivo but also an important regulatory protein in virus replication. Interestingly, this cysteine-rich C-terminal portion of V protein is expressed by all members of the *Paramyxoviridae* except HPIV-1 and HPIV-3 (11, 23), suggesting an important function associated with V protein. Whether the C-terminal portion of NDV V protein interacts with other viral or host cell proteins to modulate NDV replication and pathogenesis remains to be established.

In general, lentogenic NDVs are propagated by inoculating them into 9- to 11-day-old embryonated chicken eggs and harvesting allantoic fluid containing infectious virus 2 to 4 days after inoculation. Prolonging the incubation period to 7 days causes embryo mortality of up to 100%. During prolonged incubation, the infectious dose and the lethal dose do not differ much. In contrast to the situation with the parent virus, the use of high doses of NDV-P1 and prolonged incubation were not lethal to embryos, demonstrating that NDV-P1 is dramatically attenuated for chicken embryos (Fig. 5). The difference between the infectious and lethal doses of NDV-P1 was as high as 6.7 $\log_{10}$, compared to 0.3 $\log_{10}$ for the parent virus, showing that NDV-P1 is attenuated more than 10^6-fold (Fig. 5). Interestingly, NDV-P1 was able to cause embryo mortality when administered to embryos younger than 9 days old. The mortality reached 62% at day 7 of embryonation and decreased to 23% at day 8. NDV-P1 also reached a 10-fold-higher titer in these younger embryos than the virus grown in 9- to 11-day-old embryos. This age-dependent resistance of chicken embryos to NDV-P1 and V-deficient mutants suggests a possible role for the innate or adaptive immune response in completely preventing growth of V-deficient mutants and pathogenicity of NDV-P1 after 8 days of embryonation. It has long been known that IFN-mediated resistance of chicken embryos to viral infections increases with age (24). The phenotype of these V-defective mutants strongly suggests that they have an impaired ability to antagonize the host's innate response, in addition to having a severe replication impairment. The specific role of NDV V protein in virus replication and its involvement in counteracting innate immune responses is currently under investigation.

Recombinant SeV and MV that are defective for RNA editing and are, therefore, unable to express V protein were shown to be attenuated in vivo, although in vitro replication was not impaired (8, 17, 18, 40). Recent publications suggest that SeV and SV5 block activation of IFN-responsive genes by interacting with a cellular target, STAT1 (9, 10, 13). For SeV, the C protein was identified as being responsible for counteracting the IFN-induced antiviral state, whereas in SV5 it was the V protein that accounted for inhibiting IFN signaling by targeting STAT1 for proteasome degradation. Thus, the key determinant in SeV and SV5 pathogenicity appears to be the prevention of the IFN-mediated antiviral response. In contrast, treatment of HeLa cells with 1,000 IU of IFN produced no difference in IFN sensitivity between wild-type MV and V-deficient MV, suggesting that the IFN system probably does not play a major role in limiting the spread of MV that lacks V protein (27). It is possible that the V proteins of different members of the *Paramyxoviridae* function differently, perhaps in a host-specific manner, to overcome the antiviral effect of the immune system. In agreement with this, Didcock et al. (10) demonstrated that SV5 blocks IFN signaling in human but not in murine cells, showing that the action is host cell specific. This property may prevent one virus from crossing species barriers and causing disease in another species.

In most parts of the world, chickens and turkeys have to be protected against the ravages of ND by ND vaccines administered to hatched birds through drinking water, aerosols, or eye drops or by parenteral routes. In recent years, the in ovo technology using automated multiple-head injectors to deliver vaccines in embryonated eggs has largely replaced certain post-hatching poultry vaccines. Vaccination is generally carried out

at day 18 of embryonation and provides a labor-saving alternative to posthatching vaccination. Moreover, in ovo vaccination facilitates administration of a uniform dose of vaccine into each egg. Most posthatching NDV vaccines are based on lentogenic NDV strains that are safe for hatched chickens. Currently, however, there is no live ND vaccine that can be administered in ovo, mainly due to high embryo mortality and very low hatchability, even with the highly attenuated NDV strains. Thus, further attenuation of lentogenic NDV strains was necessary to render it safe for use as an embryo vaccine without losing immunogenicity. In the present study we succeeded in generating a recombinant NDV that is dramatically attenuated for chicken embryos. When the vaccine was administered at day 18, hatchability was not substantially affected, and hatched chickens reached body weights similar to those of control chickens (Table 2). NDV-P1 was able to induce a sufficient immune response to fully protect SPF chickens from a lethal challenge despite its reduced replication in embryonated eggs. NDV-P1 was able to confer protection not only in SPF chickens without maternally derived antibody but also in commercial chickens with high levels of maternal antibody (Tables 3 and 4). It is remarkable that NDV-P1 can provide protection in the face of the high levels of maternally derived antibody present at the time of administration and to confer protection in 85% of the chickens. The level of protection is dose dependent, and a relatively higher dose is required in commercial chickens with neutralizing maternal antibodies to achieve a high degree of protection than is required in SPF animals. Since passive immunity levels vary from flock to flock, the dose selected for practical use should remain safe in SPF chickens, in order to make sure that vaccination does not have adverse effects in animals with low levels of maternal antibody.

The attenuated mutant virus NDV-P1 not only is an attractive candidate embryo vaccine but also provides some insight into the effects of reduced levels of V protein expression in virus replication and pathogenicity. The phenotype of NDV-P1 and the inability of NDV-Δ6 and NDV-Vstop to propagate in 9- to 11-day-old chicken embryos demonstrated that genetic manipulation directed toward reducing V protein expression rather than abolishing it completely is the more promising strategy for developing a viable attenuated NDV. Such an attenuated virus is also an attractive vaccine vector for the expression of immune-stimulatory proteins or heterologous antigens derived from other poultry pathogens. Furthermore, scientific interest in NDV therapy is currently reviving, since NDV is remarkably effective in selectively killing tumor cells in humans and animals (30, 34). The possibility of generating recombinant NDV will conceivably facilitate the design of a safe and effective NDV-based anticancer therapy for humans and animals.

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Newcastle Disease Virus (NDV)-Based Assay Demonstrates Interferon-Antagonist Activity for the NDV V Protein and the Nipah Virus V, W, and C Proteins

Man-Seong Park, Megan L. Shaw, Jorge Muñoz-Jordan, Jerome F. Cros, Takaaki Nakaya,† Nicole Bouvier, Peter Palese, Adolfo García-Sastre, and Christopher F. Basler*

Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029

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We have generated a recombinant Newcastle disease virus (NDV) that expresses the green fluorescence protein (GFP) in infected chicken embryo fibroblasts (CEFs). This virus is interferon (IFN) sensitive, and pretreatment of cells with chicken alpha/beta IFN (IFN-α/β) completely blocks viral GFP expression. Prior transfection of plasmid DNA induces an IFN response in CEFs and blocks NDV-GFP replication. However, transfection of known inhibitors of the IFN-α/β system, including the influenza A virus NS1 protein and the Ebola virus VP35 protein, restores NDV-GFP replication. We therefore conclude that the NDV-GFP virus could be used to screen proteins expressed from plasmids for the ability to counteract the host cell IFN response. Using this system, we show that expression of the NDV V protein or the Nipah virus V, W, or C proteins rescues NDV-GFP replication in the face of the transfection-induced IFN response. The V and W proteins of Nipah virus, a highly lethal pathogen in humans, also block activation of an IFN-inducible promoter in primate cells. Interestingly, the amino-terminal region of the Nipah virus V protein, which is identical to the amino terminus of Nipah virus W, is sufficient to exert the IFN-antagonist activity. In contrast, the anti-IFN activity of the NDV V protein appears to be located in the carboxy-terminal region of the protein, a region implicated in the IFN-antagonist activity exhibited by the V proteins of mumps virus and human parainfluenza virus type 2.

The alpha/beta interferon (IFN-α/β) system is a major component of the host innate immune response to viral infection (reviewed in reference 1). IFN (i.e., IFN-β and several IFN-α types) is synthesized in response to viral infection due to the activation of several factors, including IFN regulatory factor proteins, NF-κB, and AP-1 family members. As a consequence, viral infection induces the transcriptional upregulation of IFN genes. Secreted IFNs signal through a common receptor activating a JAK/STAT signaling pathway which leads to the transcriptional upregulation of numerous IFN-responsive genes, a number of which encode antiviral proteins, and leads to the induction in cells of an antiviral state. Among the antiviral proteins induced in response to IFN are PKR, 2′,5′-oligoadenylate synthetase (OAS), and the Mx proteins (10, 15, 23).

Many viruses have evolved mechanisms to counteract the host IFN response and, in some viruses, including vaccinia virus, adenovirus, and hepatitis C virus, multiple IFN-antagonist activities have been reported (3, 6, 12, 16, 17, 28, 35, 57, 58). Among negative-strand RNA viruses, several different IFN-subverting strategies have been identified that target a variety of components of the IFN system. The influenza virus NS1 protein, for example, prevents production of IFN by inhibiting the activation of the transcription factors IFN regulatory factor 3 and NF-κB and blocks the activation of the IFN-induced antiviral proteins PKR and OAS (4, 18, 55, 59; N. Donelan, X. Wang, and A. García-Sastre, unpublished data). Among the paramyxoviruses, different mechanisms are employed by different viruses (60). For example, the "V" proteins of several paramyxoviruses have previously been shown to inhibit IFN signaling, but the targets of different V proteins vary (32, 47). In the case of Sendai virus, the "C" proteins, a set of four carboxy-coterminal proteins, have been reported to block IFN signaling both in infected cells and when expressed alone (19, 21, 22, 27, 30). In contrast, respiratory syncytial virus, which encodes neither a C nor a V protein, produces two nonstructural proteins, NS1 and NS2, that are reported to cooperatively counteract the antiviral effects of IFN (5, 54). Ebola virus, a nonsegmented, negative-strand RNA virus of the family *Filoviridae* that possesses a genome structure similar to that of the paramyxoviruses (29), also encodes at least one protein, VP35, that counteracts the host IFN response (2).

Viral IFN antagonists have been shown to be important virulence factors in several viruses, including herpes simplex virus type 1, vaccinia virus, influenza virus, and Sendai virus. Analysis of viruses with mutations in genes encoding herpes simplex virus type 1 ICP34.5 (8, 38), vaccinia virus E3L (6), influenza virus NS1 (18, 56), and Sendai virus C (13, 20) proteins has demonstrated an important role for each of these IFN antagonists in viral pathogenicity in mice. Because IFN antagonists are important virulence factors, their identification and characterization should provide important insights into viral pathogenesis.

Infectious cDNAs for Newcastle disease virus (NDV) have recently been developed (31, 42, 49, 51) and permit the introduction of foreign genes into the NDV genome (31, 42, 53). We constructed a recombinant NDV expressing the green flu-

* Corresponding author. Mailing address: Department of Microbiology, Box 1124, Mount Sinai School of Medicine, One Gustave L. Levy Pl., New York, NY 10029. Phone: (212) 241-5923. Fax: (212) 534-1684. E-mail: chris.basler@mssm.edu.

† Present address: Department of Microbiology, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan.

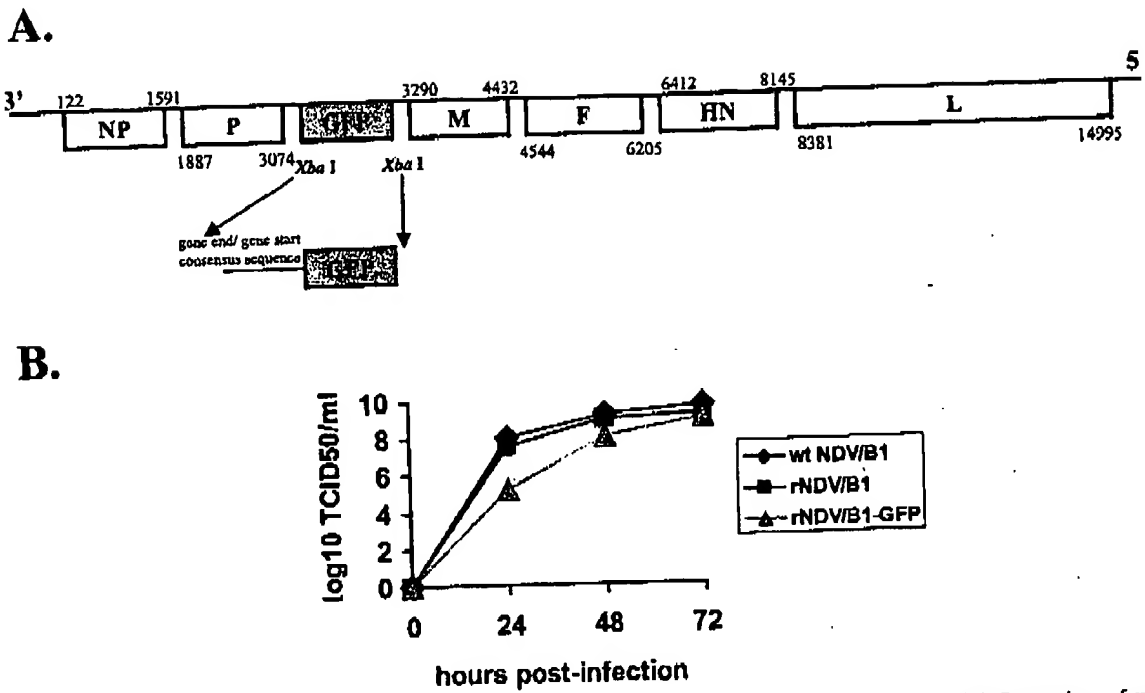


FIG. 1. Construction and growth of an NDV expressing GFP. (A) Diagram of the NDV-GFP virus genome. (B) Comparison of growth of recombinant NDV Hitchner B1 and NDV-GFP viruses in CEFs (MOI = 0.001).

orescence protein (GFP), NDV-GFP, and show that this virus is sensitive to the antiviral effects of IFN. We have taken advantage of this IFN-sensitive property and developed an NDV-GFP-based assay to identify proteins that exhibit IFN-antagonist activity. Using this system, we provide evidence that the NDV V protein possesses IFN-antagonist activity. We further use this assay to show that the V, W, and C proteins of Nipah virus, an important emerging pathogen that is highly lethal in humans (9, 14, 34), also exhibit IFN-antagonist activity.

MATERIALS AND METHODS

Cells and plasmids. Chicken embryo fibroblasts (CEFs) were prepared from 10-day-old specific-pathogen-free embryos (Charles River SPAFAS, North Franklin, Conn.). CEFs and Vero cells were maintained in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) and Dulbecco modified Eagle medium with 10% FBS, respectively. The influenza virus NS1 and the Ebola virus VP35 protein expression plasmids have been described previously (2, 55).

Construction and growth of a GFP-expressing NDV. The enhanced GFP open reading frame (ORF) from the plasmid pEGFP-c1 (Clontech) was cloned between the P and M genes of the previously described NDV Hitchner B1 cDNA (42) (Fig. 1). This virus, NDV-GFP, was then rescued from cDNA by using previously described methods (42), and the presence in the viral genome of the inserted GFP gene was confirmed by reverse transcription-PCR and sequencing (data not shown). Virus stocks were prepared in 10-day-old embryonated chicken eggs. Virus titers were determined as $TCID_{50}$/ml on CEFs. Briefly, 96-well plates containing CEFs were infected with 10-fold serial dilutions of virus. At 2 days postinfection, cells were fixed with 2.5% formaldehyde containing 0.1% Triton X-100. Infection of individual wells was determined by indirect immunofluorescence with polyclonal, anti-NDV rabbit antiserum as the primary antibody and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin (Dako Corp.).

Cloning of the NDV V ORF and the Nipah virus V, W, and C ORFs. The NDV V ORF was constructed by PCR amplification from the wild-type NDV Hitchner B1 P protein expression plasmid pCAGGS-NDV P (42). The NDV V ORF was amplified as two PCR fragments by using the following primers. For the amino-terminal fragment, the sense primer 5′-CC GAA TTC ATGGCC ACC TTT ACA GAT, containing an *Eco*RI site (underlined), and the antisense primer 5′-GGC TCG ACC ATG GGC CCC TT, containing an *Nco*I restriction site (underlined), were used. For the carboxy-terminal fragment, the sense primer 5′-AAG GGG CCC ATG GTC GAG CC and the antisense primer 5′-CG CTC GAG TTA CTT ACT CTC TGT GAT ATC, each containing an *Xho*I restriction site (underlined), were used. For the NDV V_N ORF, the sense primer 5′-CC GAA TTC ATGGCC ACC TTT ACA GAT and the antisense primer 5′-CG CTC GAG TCA TTT AGC ATT GGA CGA TTT were used. For the NDV V_C ORF, the sense primer 5′-CC GAA TTC CCC ATG GTC GAG CCC CCA and the antisense primer 5′-CG CTC GAG TTA CTT ACT CTC TGT GAT ATC were used.

The Nipah virus cDNAs were constructed by PCR without template by using overlapping deoxyoligonucleotides corresponding to GenBank accession number NC_002728. The V ORF corresponds to nucleotides 2406 to 3775 but contains a single non-template-encoded G residue inserted after position 3624. The W ORF corresponds to nucleotides 2406 to 3756 but contains two non-template-encoded G residues inserted after position 3624. The C ORF corresponds to nucleotides 2428 to 2928. All ORFs were cloned into the mammalian expression plasmid pCAGGS (44). Because the C ORF lies within the V and W ORFs, it could conceivably be produced from the Nipah virus V and W expression plasmids if the first ATG in these plasmids were bypassed. Therefore, all plasmids expressing V or W proteins contained mutations in which the two tandem ATGs at the predicted start of the C protein were mutated to ACG. These mutations would be expected to eliminate or significantly reduce the level of full-length C protein that might be expressed. Additionally, a V protein expression plasmid was generated in which the two tandem ATGs at the predicted start of the C protein were mutated to ACG and a stop codon was introduced at the fourth codon of the C ORF. The changes did not affect the translation of the V protein. Primer sequences and PCR conditions are available upon request.

Transfection and infection of CEF cells. One day prior to transfection, CEFs were seeded onto 24-well plates such that they would be 80% confluent the following morning. The medium was replaced with 0.3 ml of Dulbecco modified Eagle medium with 5% FBS. Transfection mixtures were prepared in polystyrene tubes as follows. First, 2 μg of plasmid DNA was diluted to 50 μl in OptiMEM

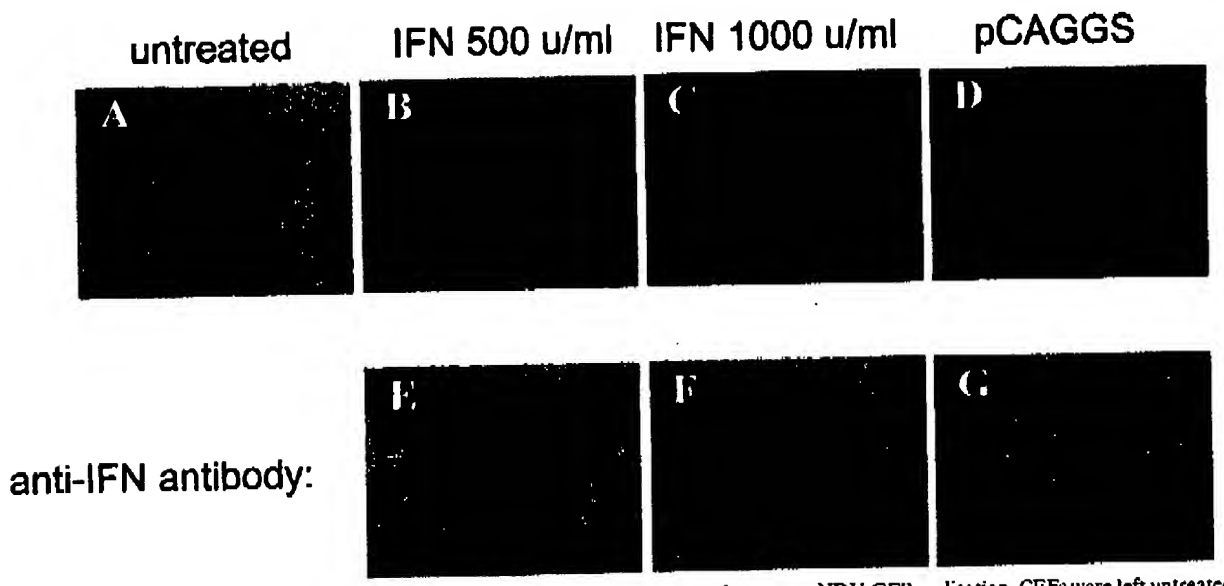


FIG. 2. Transfection of CEFs with plasmid DNA induces production of IFN-α/β and represses NDV-GFP replication. CEFs were left untreated (A), were treated with chicken IFN-α/β at 500 U (B) or 1,000 U (C)/ml, or were transfected with the empty expression plasmid pCAGGS (D). IFN treatment was performed in the absence (A to C) or presence (E and F) of neutralizing antibody against chicken IFN. Transfection of cells with empty vector was performed in the absence of anti-IFN antibody (D), or else the antibody was added immediately after the transfection (G). Cells were infected with NDV-GFP at an MOI of 1 at 20 h posttreatment or posttransfection and then examined for green fluorescence at 24 h postinfection.

(Gibco). Then, 6 μl of Fugene 6 (Roche) previously diluted to 50 μl in OptiMEM was added to the polystyrene tube. The transfection mixtures were incubated for 20 min at room temperature and added to the cells. After 20 h of incubation at 37°C in 5% CO_2, the transfected cells were washed briefly with phosphate-buffered saline and infected with NDV-GFP (multiplicity of infection [MOI] of 1 to 2) at room temperature for 30 to 60 min. The inoculum was then aspirated and 0.5 ml of MEM–10% FBS was added to the cells. The infected cells were incubated for 20 h at 37°C in 5% CO_2 prior to detection of green fluorescence.

Neutralization of chicken IFN. Chicken IFN-α/β (1,000 or 500 U) was added to cells 8 h after transfection or to nontransfected cells where specified. Neutralizing mouse antibody against chicken IFN was added together with IFN or immediately after transfection where specified. The chicken IFN and the anti-chicken IFN antibodies were kindly provided by Peter Stueheli (University of Freiburg) and Bernd Kaspers (University of Munich).

FACS analysis. Cells either mock-infected or infected with NDV-GFP were detached from dishes by treating the cells with trypsin-EDTA and resuspending them in 1 ml of MEM–10% FBS. The relative mean intensity of green fluorescence of the cells was determined by fluorescence-activated cell-sorting (FACS) analysis with a Beckman-Coulter Epics XL-MCL fluorescence-activated cell sorter.

Reporter gene assays in Vero cells. Vero cells were transfected with three plasmids: a pCAGGS construct of the gene of interest, a plasmid containing the chloramphenicol acetyltransferase (CAT) gene downstream of an IFN-stimulated response element (ISRE) (pHISG-54-CAT), and a construct encoding the *Renilla* luciferase protein (pRL-tk). The cells were transfected with 1 μg of each reporter plasmid and 5 μg of the expression plasmid by using the transfection reagent Lipofectamine 2000 (Invitrogen). The following day the medium was replaced with medium containing 1,000 IU of IFN-β/ml, and incubation was continued overnight. The cells were harvested, and the cell pellet was resuspended in phosphate-buffered saline and divided into two aliquots for analysis of the CAT and luciferase activities. CAT assays were performed as previously described (50a). Luciferase assays were performed by using the *Renilla* luciferase assay system (Promega) according to the manufacturer's instructions.

RESULTS

Construction of an NDV that expresses GFP. The GFP ORF was cloned between the P and M genes of the previously described NDV Hitchner B1 cDNA (42) (Fig. 1A). This virus, NDV-GFP, was then rescued from cDNA by previously described methods, and the presence of the inserted GFP gene in the viral genome was confirmed by reverse transcription-PCR and sequencing (data not shown). The growth of this virus on CEFs was compared to that of the parental recombinant Hitchner B1 strain and to the original NDV Hitchner B1 virus used to construct the infectious cDNA (Fig. 1B). Growth of the GFP-expressing virus was similar to that of the viruses without the GFP insert. As expected, infected CEFs displayed strong green fluorescence at 1 day postinfection (Fig. 2A).

Transfection of CEFs with plasmid DNA induces production of IFN and represses NDV-GFP replication. We examined the effect of treating CEFs with chicken IFN prior to NDV-GFP infection. Infection of untreated CEFs resulted in GFP expression (Fig. 2A). When cells were treated with 500 or 1,000 U of chicken IFN-α/β per ml 1 day prior to infection, viral GFP expression was greatly reduced, suggesting that viral replication was significantly impaired (Fig. 2B and C). However, viral GFP expression was restored when a neutralizing anti-chicken IFN antibody was added 8 h after addition of IFN (Fig. 2E and F). We also found that prior transfection of cells with plasmid DNA blocked GFP expression, and this inhibition was even more pronounced than when cells were pretreated with 1,000 U of IFN/ml (Fig. 2D). GFP expression could be restored in transfected and infected cells by addition of anti-IFN antibody to the cell culture supernatant, suggesting that DNA transfection inhibits NDV-GFP replication largely, if not exclusively, through the induction of IFN production (Fig. 2G). We have also established that cell supernatants taken from transfected cells and transferred to fresh CEFs inhibit NDV-GFP replication (data not shown). Interestingly, the induction of an IFN

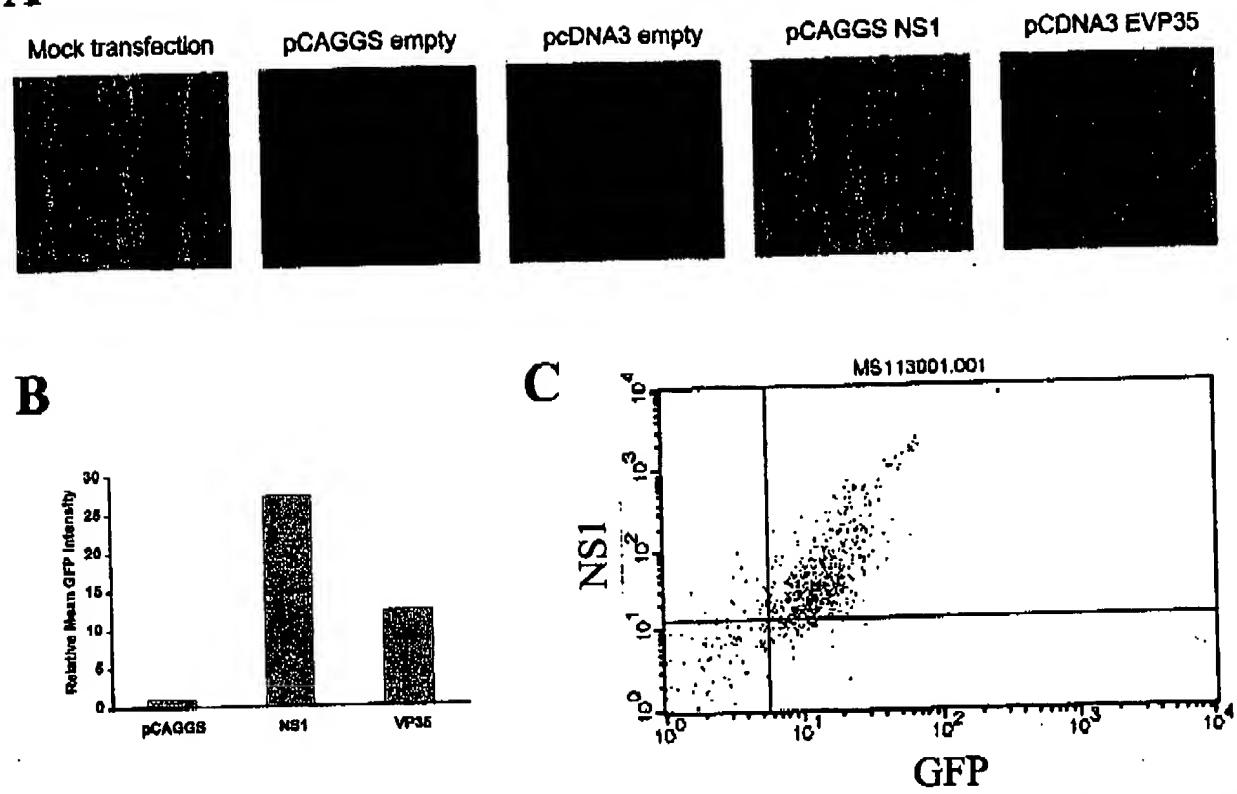


FIG. 3. Expression of the influenza virus NS1 or Ebola virus VP35 proteins prevents plasmid transfection-induced inhibition of NDV-GFP replication. (A) GFP expression in NDV-GFP-infected cells transfected 24 h prior to infection with various plasmids. Cells were mock transfected, transfected with empty pCAGGS or pcDNA3 expression plasmids, or transfected with expression plasmids for the influenza A virus NS1 protein or the Ebola virus VP35 protein as indicated. (B) Mean relative green fluorescence intensity of CEFs transfected with the indicated plasmids (pCAGGS, pCAGGS-NS1, or pcDNA3-VP35) and subsequently infected with NDV-GFP. The graph shows the mean relative intensity of green fluorescence for 10^4 cells from each culture as determined by FACS. The results from panel A and panel B are from the same experiment and are representative of typical results with the indicated plasmids. (C) Expression of NS1 in green fluorescent cells. CEFs were transfected with NS1 expression plasmid and infected with NDV-GFP 24 h posttransfection. At 24 h postinfection, the cells were fixed, permeabilized, and stained with rabbit antiserum against the influenza A virus NS1 protein. FACS analysis was then performed to detect both NS1 (*y* axis) and GFP (*x* axis).

response in CEFs appears to require both the transfection reagent and DNA. Inhibition of NDV-GFP expression was not seen when only transfection reagent or only DNA was added to cells (data not shown). The requirement for both the transfection reagent and the plasmid DNA to induce IFN production suggests that transcription from the transfected plasmids is the actual trigger of IFN production. Transcription from the mammalian expression plasmids can lead to the production of double-stranded RNA, a factor known to induce IFN production (37).

Expression of the IFN-antagonist influenza virus NS1 or Ebola virus VP35 proteins prevents plasmid transfection-induced inhibition of NDV-GFP replication. The influenza virus NS1 protein and the Ebola virus VP35 protein have previously been shown to inhibit cellular IFN responses (2, 18, 55, 59). Transfection of plasmids encoding either of these IFN antagonists could overcome the transfection-mediated inhibition of NDV-GFP replication. As seen previously, transfection of empty vector (either pCAGGS or pcDNA3) 1 day before infection prevented GFP expression from NDV-GFP-infected cells (Fig. 3A). However, when either NS1 or VP35 expression plasmids were transfected, GFP expression could be readily detected 1 day postinfection (Fig. 3A). When the intensity of GFP expression was analyzed by FACS, the enhancement of green fluorescence in NS1- or VP35-expressing cells was clearly demonstrated (Fig. 3B). When NS1-expressing, NDV-GFP-infected cells were fixed and stained with anti-NS1 antiserum and then analyzed by FACS, the levels of GFP expression were found to correlate with the levels of NS1 expression (Fig. 3C). These data demonstrate that expression of IFN antagonists prevents transfection-induced inhibition of NDV-GFP replication and provide further evidence that the transfection-mediated inhibition of NDV-GFP growth is related to activation of the cellular IFN response. These experiments also suggest that the NDV-GFP assay can be used as a screen for proteins with IFN-antagonist activity.

Expression of the NDV V protein prevents transfection-

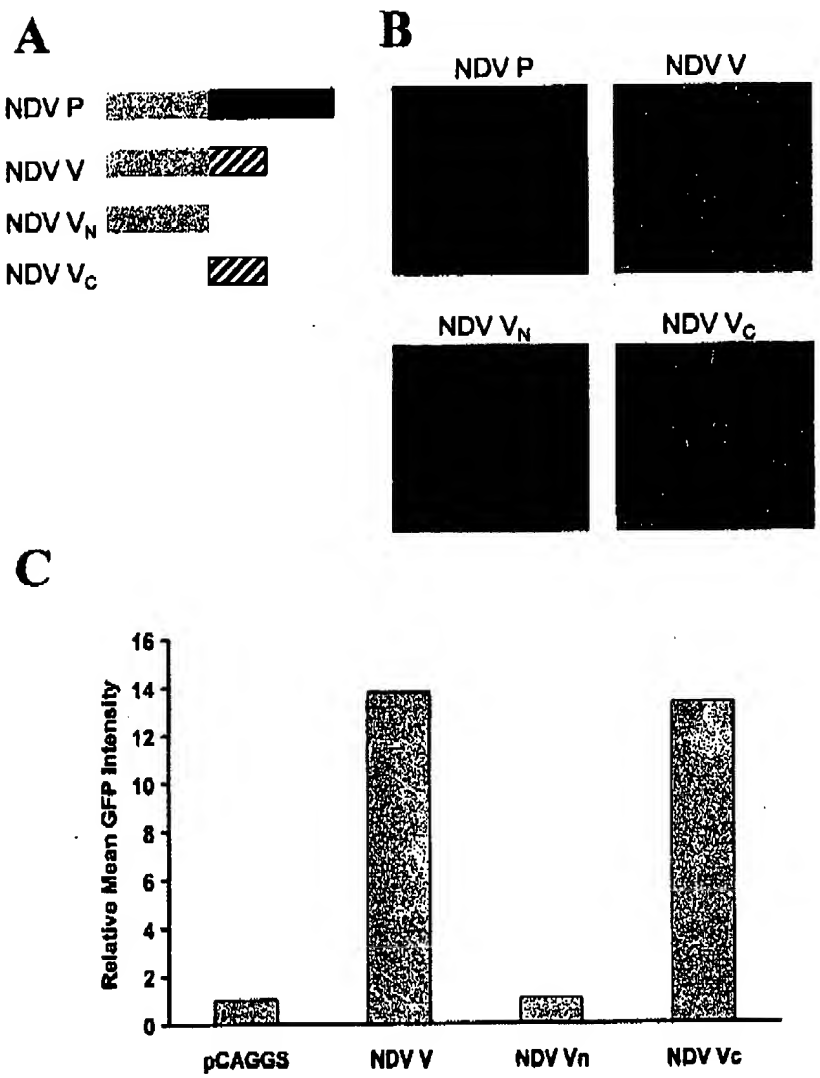


FIG. 4. Expression of the NDV V protein prevents transfection-induced inhibition of NDV-GFP replication. (A) Diagram of the four NDV P and V constructs used. The gray boxes on the left indicate shared amino-terminal domains. The V protein possesses a cysteine-rich carboxy-terminal region distinct from the P protein. This V-specific domain arises due to the insertion of a single non-template-encoded G residue and is indicated by the hatched box. V_N possesses only the shared amino-terminal region. V_C possesses the V carboxy terminus to which an initiator ATG has been added. (B) GFP expression in NDV-GFP-infected cells transfected 24 h prior to infection with NDV P, NDV V, NDV V_N, or NDV V_C expression plasmids. (C) Relative mean green fluorescence of CEFs transfected with empty vector (pCAGGS) or transfected with NDV P, NDV V, NDV V_N, or NDV V_C expression plasmids as indicated and subsequently infected with NDV-GFP. Shown is the relative mean intensity of green fluorescence for 10^4 cells from each culture as determined by FACS. The results from panels B and C are from the same experiment and are representative of typical results with the indicated plasmids.

induced inhibition of NDV-GFP replication. Because NDV encodes a V protein but not a C protein and because the V proteins of several paramyxoviruses display IFN-antagonist activity, the V protein of NDV was tested for its ability to rescue the growth of NDV-GFP virus. Transfection of an empty plasmid once again inhibited NDV-GFP replication. In contrast, transfection of CEF cells with an NDV V protein expression plasmid restored the ability of NDV-GFP to grow (Fig. 4A and B). It should be noted that although the NDV-GFP virus possesses an intact V ORF, the IFN-induced inhibition of viral growth occurs prior to infection, allowing an antiviral state to be established before V protein is expressed from virus.

The NDV V protein is produced in infected cells from an edited transcript in which the viral polymerase has added a single non-template-encoded G residue. As a result, the amino-terminal region of V is identical to the amino terminus of P. However, after the editing site, the proteins are different (Fig. 4A). In order to determine which region of V was responsible for the restoration of GFP expression, three additional constructs were tested. These plasmids encode the full-length P

protein, the amino-terminal region of V which is shared in common with the P protein or the carboxy-terminal domain of V which is distinct from P (Fig. 4A). Whereas the carboxy-terminal domain of V and the full-length V were able to rescue GFP expression, neither the V_N nor the P constructs rescued NDV-GFP growth (Fig. 4B). Analysis of the relative intensity of green fluorescence from this experiment demonstrated that cells expressing either full-length NDV V or the carboxy-terminal domain resulted in an average GFP fluorescence intensity approximately 13 times that of mock-transfected, infected cells. (Fig. 4C). These data indicate that the observed IFN-antagonist activity is encoded by the carboxy terminus of NDV V.

The Nipah virus V, W, and C proteins display IFN-antagonist activity in the NDV-GFP system. The NDV-GFP assay was then used to screen several Nipah virus protein expression plasmids for IFN-antagonist activity. Nipah virus was chosen because of its importance as an emerging paramyxovirus that causes severe disease in humans (9). Because this virus is expected to produce V, W, and C proteins from its P gene (7; B. H. Harcourt, A. Tamin, B. Newton, A. Sanchez, T. G. Ksiazek, P. E. Rollin, W. J. Bellini, and P. A. Rota, Abstr. 11th Int. Conf. Negative Strand Viruses, abstr. 170, 2000), we tested the V, W, and C ORFs in the NDV-GFP assay (Fig. 5A). Expression of the Nipah virus V protein rescued the growth of NDV-GFP (Fig. 5B and C). Likewise, a W protein expression plasmid was also found to enhance NDV-GFP replication (Fig. 5B and C). The V and W plasmids used for these experiments were altered to decrease the possibility that they would also express the C protein, which is encoded entirely within the amino terminus of the V or W proteins. The two tandem ATGs predicted to begin the C ORF were mutated to ACG. These mutations were expected to reduce or eliminate C protein expression. Although ACG is used as a start codon to initiate translation of the Sendai virus C′ protein, it is an inefficient start codon (11). We have also tested an additional V protein expression plasmid in which the C gene was mutated not only at these two ATGs but also by introduction of a stop codon at position four of the C ORF. These changes did not affect V protein translation, and this C knockout construct also rescued NDV-GFP growth (data not shown). These data suggest that the enhancement of NDV-GFP replication seen with V and W expression plasmids occurs independent of C protein expression.

It was striking that both V and W exerted an anti-IFN effect in this system. Because these proteins possess the same 407 amino-terminal amino acids, this common region was also tested and found to also enhance green fluorescence in NDV-GFP-infected cells (Fig. 5B and C). Interestingly and in contrast to the data obtained with the NDV V, the carboxy-terminal region of Nipah virus V, which possesses the relatively conserved cysteine-rich region, did not show significant IFN-antagonist activity in this assay (Fig. 5B and C).

Because the C proteins of Sendai virus have been reported to counter the host IFN response, we also tested the Nipah virus C protein in the NDV-GFP assay. When a Nipah virus C protein expression plasmid was transfected, GFP expression was also detected, although the intensity of the green fluorescence in C-transfected cells was typically lower than that seen with the V or W constructs (Fig. 5D and E).

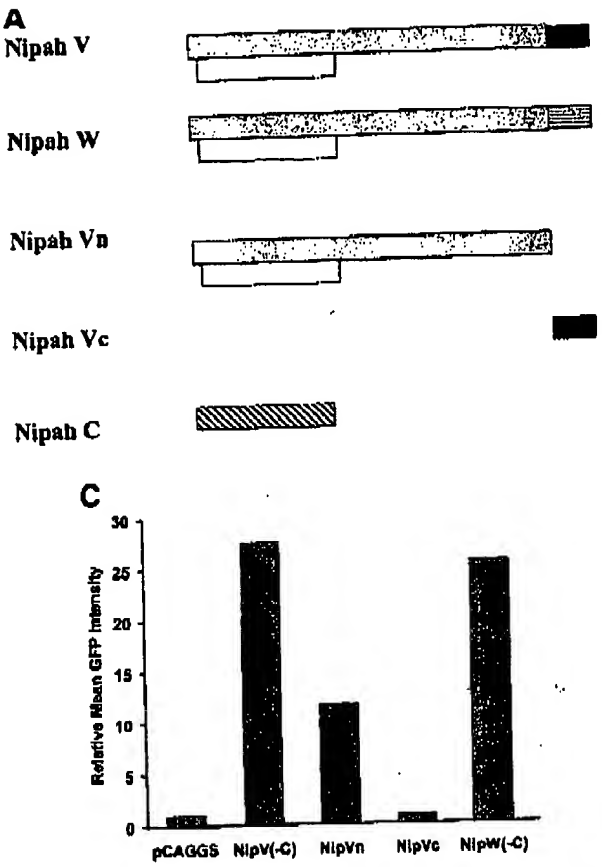
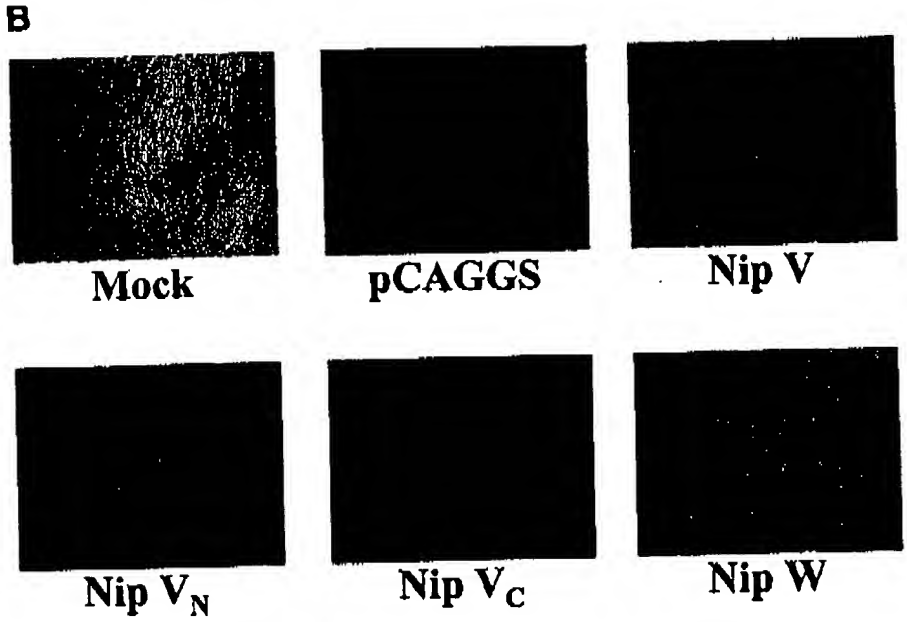
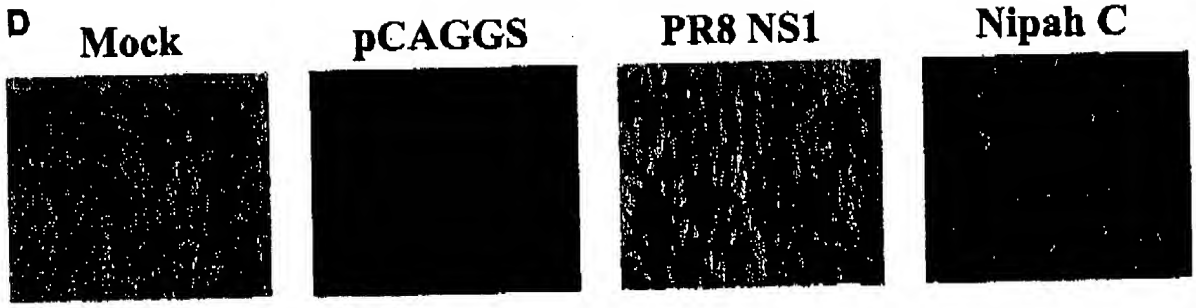


FIG. 5. (A) Diagram of the five Nipah P, V, and W constructs used. The shaded boxes indicate the shared amino-terminal domains. The V protein possesses a cysteine-rich carboxy-terminal region distinct from the P protein. This V-specific domain arises due to the insertion of a single non-template-encoded G residue and is indicated by the solid box. The W protein possesses a carboxy-terminal region distinct from both the P and the V proteins. This W-specific domain arises due to the insertion of two non-template-encoded G residues and is indicated by the box with horizontal lines. V_N possesses only the shared amino-terminal region. V_C possesses the V carboxy terminus, to which an initiator ATG has been added. The C ORF is indicated by the hatched box. Because the ATGs at the beginning of the C ORF have been mutated in the V, W, and V_N constructs, the C ORF in these constructs is indicated by the open box. (B) GFP expression in NDV-GFP-infected cells that were, 24 h prior to infection, mock transfected or transfected with the empty pCAGGS plasmid or expression plasmids for Nipah virus V (Nip V_N), the amino-terminal domain of Nipah virus V (Nip V_N), the carboxy-terminal domain of V (Nip V_C), or Nipah virus W (Nip W). (C) Relative mean green fluorescence of CEFs transfected with empty vector (pCAGGS) or transfected with the plasmids encoding Nip V, Nip V_N, Nip V_C, or Nip W. These plasmids were engineered so as not to express the C protein [indicated by (−C)]. Shown is the relative mean intensity of green fluorescence for 10^4 cells from each culture as determined by FACS. (D) GFP expression in NDV-GFP-infected cells that were, 24 h prior to infection, mock transfected or transfected with the empty pCAGGS plasmid or expression plasmids for influenza virus NS1 or Nipah virus C proteins. (E) Relative mean green fluorescence of CEFs transfected with empty vector (pCAGGS) or transfected with the plasmids encoding influenza virus NS1 or Nipah virus C. Shown is the relative mean intensity of green fluorescence for 10^4 cells from each culture as determined by FACS. The results from panels B and C are from the same experiment, and the results from panels D and E are from the same experiment and are representative of typical results with the indicated plasmids.





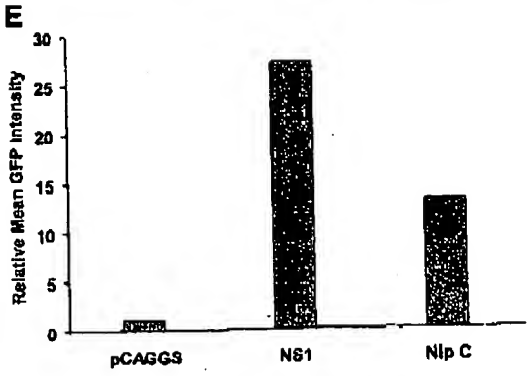


FIG. 5—*Continued.*

The Nipah virus V and W proteins inhibit IFN-β-mediated activation of an IFN responsive promoter. The V proteins of human parainfluenza virus type 2 (hPIV-2), simian virus 5 (SV5), and mumps virus reportedly inhibit the IFN signaling pathways (32, 47). We therefore determined whether expression plasmids encoding the Nipah virus V or W or truncated forms of Nipah virus V inhibited, in Vero cells, the activation by IFN-β of an IFN-inducible promoter (Fig. 6). Transfection of plasmids possessing the entire V ORF, the entire V ORF in which the C ORF was disrupted, or the entire W ORF resulted in a near-complete block in ISRE reporter gene expression in response to IFN (Fig. 6). Further, a plasmid containing only the amino-terminal domain common to V and W also blocked reporter gene activation, whereas a plasmid containing only the unique carboxy-terminal region of V failed to block reporter gene activation (Fig. 6). These data are consistent with the IFN-antagonist activity seen with these proteins in the NDV-GFP assay and suggest that the common amino-terminal domain of Nipah virus V and W can function to block the IFN signaling pathway. When the Nipah virus C expression plasmid which enhanced NDV-GFP replication in CEFs was tested in the reporter gene assay in Vero cells, it had a much less dramatic effect on IFN-induced reporter gene activation. Nipah virus C was found to reproducibly decrease reporter gene activity to a modest degree (data not shown). It is unclear whether this modest inhibition of IFN signaling is sufficient to permit NDV-GFP replication or whether Nipah virus C may enhance NDV-GFP replication by an alternative mechanism.

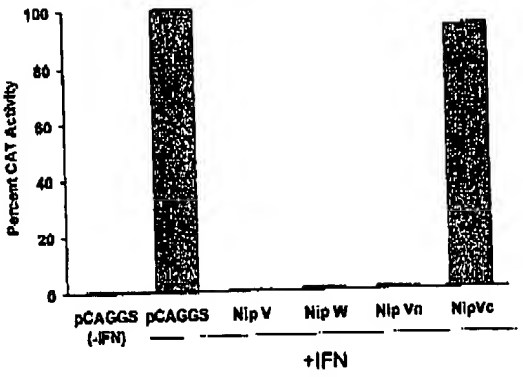


FIG. 6. Inhibition in Vero cells of IFN-induced activation of an ISRE promoter by Nipah virus proteins. Effect of Nipah virus V, W, and V truncation mutant expression plasmids on IFN-β-induced activation of an IFN-inducible promoter. Vero cells were transfected with the indicated plasmids and either mock treated (−IFN) or treated with human IFN-β (+IFN). The expression plasmids encoded Nipah virus V (Nip V), Nipah virus W (Nip W), the amino-terminal region common to Nipah virus V and W (Nip Vn), or the carboxy-terminal region of Nipah virus V specific to V (Nip Vc). Shown is the relative activity of an IFN-inducible CAT reporter gene under the control of the IFN-inducible HISG54 promoter normalized to an internal control consisting of a constitutively expressed *Renilla* luciferase expression plasmid. The cells were treated with 1,000 U of human IFN-β/ml at 1 day posttransfection, and CAT and luciferase assays were performed 1 day later.

DISCUSSION

Using an NDV-GFP-based assay, we found that expression of the NDV V protein or of the (putative) Nipah virus V, W, and C proteins can prevent establishment of an IFN-induced antiviral state. The NDV-GFP assay provides a straightforward system by which cloned viral genes can be screened for IFN-antagonist activity. NDV-GFP is a virus that is susceptible to the antiviral effects of IFN, although it encodes a functional IFN antagonist, the V protein. It thus appears that the presence of a functional NDV V gene may not be sufficient to overcome the previously established antiviral state within the time frame of the assay. This contention is supported by the following observation: when CEF cells are treated once with IFN-α/β at 20 h prior to infection, NDV-GFP replication is undetectable, although green fluorescence becomes detectable at 48 h postinfection under these conditions (data not shown). Likewise, prior transfection of CEF cells with plasmid DNA results in the secretion of IFN, and this IFN response suppresses, for at least 24 h, the replication of subsequently added NDV-GFP. It is likely that the transfection produces sufficient IFN that an antiviral state is well established before NDV-GFP infection. However, when cells are transfected with plasmids expressing IFN antagonists, including the influenza virus NS1 protein or the Ebola virus VP35 protein, the induction of an antiviral state can be reduced or prevented, and GFP expression is easily detected less than 24 h postinfection.

One advantage of the NDV-GFP assay is the simple and rapid readout. It should be noted that the absolute number and intensity of green cells seen from experiment to experiment are variable, but the relative activity of the various transfected proteins is consistent between experiments. Additionally, the levels of NDV-GFP replication vary depending on which IFN antagonist is expressed. The reason(s) that one protein enhances NDV-GFP replication to a different degree than another is not clear but might be related to different levels of expression or to different mechanisms of action (e.g., one may target the JAK/STAT signaling pathway, whereas another might prevent the production of IFN). Another helpful aspect of the system is that the test virus (NDV-GFP) readily grows to high titers in 10-day-old embryonated chicken eggs. It remains to be seen whether the use of chicken cells limits the usefulness of the assay. In this respect, it is encouraging that the influenza virus NS1 protein, the Ebola virus VP35 protein, and the Nipah virus proteins all appear to function as IFN antagonists in both CEFs and mammalian systems. This is despite the fact that some paramyxovirus V proteins appear to function in a species-specific manner (46). We also have preliminary data indicating that the NDV-GFP assay can be adapted to at least some mammalian cells. The use of different cell lines may help in the identification of IFN antagonists that function in a cell type-dependent fashion.

Paramyxovirus V, W, and C proteins are encoded by the viral phosphoprotein (P) gene. The P protein and the V protein always share a common amino terminus but possess unique carboxy termini due to the insertion of a non-template-encoded G residue(s) at a precise point during transcription of the P gene, a process called "editing." In some paramyxoviruses, including all members of the *Rubulavirus* genus except for NDV, the V protein is encoded by the unedited mRNA (33). For the remaining paramyxoviruses that encode V proteins, V is encoded by an edited transcript in which one non-template-encoded G has been inserted (33). In addition, some paramyxoviruses produce additional edited P-gene transcripts that encode proteins with amino-terminal sequences identical to that of the P protein. These include "W" proteins, such as that predicted for Nipah virus (Harcourt et al., 11th Int. Conf. Negative Strand Viruses), which would arise from edited mRNAs in which two G residues are inserted at the editing site into the P gene mRNA. The C proteins are encoded by the P transcripts as well but arise due to the use of alternate start codons and do not possess amino acid identity with the P protein (33). As with V proteins, not all paramyxoviruses encode C proteins, although some, such a Nipah virus, encode C proteins, V proteins, and additional P gene-derived proteins (e.g., W proteins) (33).

It will be important to determine the specific mechanisms by which the NDV V and the Nipah virus V, W, and C proteins counter the IFN response. In this regard, the Nipah virus V protein has recently been reported to cause cytoplasmic retention of STAT1 and STAT2 (50a). Among other paramyxoviruses, different viruses have been shown to employ different mechanisms to overcome the host IFN response (5, 19, 21, 22, 27, 30, 32, 46, 47, 54, 60). Several paramyxovirus V proteins have previously been shown to inhibit IFN signaling. For example, the mumps virus V protein has been reported to decrease cellular STAT1 levels, possibly by targeting STAT1 for proteosome-mediated degradation (32). Similarly, the "V" proteins of SV5 and hPIV-2 also appear to promote STAT protein degradation although, whereas SV5 V promotes STAT1 degradation, hPIV-2 V promotes the degradation of STAT2 (47). Interestingly, V-mediated degradation of one

specific STAT (STAT1 or STAT2) protein required the presence of the second STAT (47), and the inability of SV5 V to promote the degradation of STAT1 in mouse cells was related to the absence of a compatible (to the SV5 V protein) STAT2 protein (46). Our data indicate that the NDV V also blocks the host cell IFN response, although its mechanism of action has yet to be defined. The ability of the Nipah virus V or W protein expression plasmids to not only rescue NDV-GFP growth but also prevent activation of the IFN-inducible reporter genes in response to IFN-β treatment suggests that the Nipah virus V and W proteins will also affect some component of the IFN-α/β signaling pathway. It is interesting that the Nipah virus C expression plasmid also showed an ability to rescue NDV-GFP replication but displayed only a weak inhibition in the IFN-induced reporter gene system. In the case of Sendai virus, the "C" proteins, a set of four carboxy-coterminal proteins, have been reported to block IFN signaling both in infected cells and when expressed individually (19, 21, 22, 27, 30, 52). It is therefore unclear whether the IFN-antagonist activity of the Nipah virus C seen in the NDV-GFP assay can be fully accounted for by a weak block in the IFN signaling pathway or whether Nipah virus C has additional biological functions.

It was striking that different regions of the NDV V and the Nipah virus V proteins were required for anti-IFN activity. The carboxy-terminal domain of the various paramyxovirus V proteins is relatively conserved and includes seven cysteine residues that together form a zinc finger (24, 36, 45, 48). As was the case for the NDV V in the present study, the conserved, carboxy-terminal domains of either the mumps virus V protein or the hPIV-2 V protein were capable of blocking the host cell IFN signaling pathway (32, 43). In contrast, in both the NDV-GFP assay (Fig. 5) and the reporter gene assay (Fig. 6), IFN-antagonist activity was clearly associated with the amino-terminal region common to the Nipah virus P, V, and W proteins. In contrast, little to no IFN-antagonist activity was detected with the cysteine-rich carboxy terminus of the Nipah virus V protein. However, although it is clear that the amino terminus of V is sufficient for IFN-antagonist activity in these assays, it remains possible that the carboxy-terminal domain of Nipah virus V contributes to the IFN-antagonist function of the full-length Nipah virus V protein or that the carboxy terminus of V will display a species-specific IFN-antagonist activity. It is interesting that the V, W, and P proteins of Nipah virus and Hendra virus possess longer amino termini than do other paramyxoviruses (7). Perhaps the unique 210 amino-terminal amino acids common to the Nipah virus V and W proteins possess the IFN-antagonist activity. It is also interesting that the Ebola virus VP35 protein is functionally analogous to the paramyxovirus P proteins and also counteracts the host IFN response (Fig. 2) (2, 40, 41). However, filovirus VP35 proteins do not appear to encode C, V, or W protein equivalents. Thus, it is clear that P genes (or their equivalents) of negative-strand RNA viruses have frequently evolved IFN-antagonist functions. In cases such as Ebola virus, the P protein itself may be sufficient to carry out this function. In other viruses, such "auxiliary" functions may have been shifted exclusively to C or V proteins during the course of evolution. In the case of Nipah virus, although V and W proteins are produced and exert an anti-IFN function, it remains possible that the P protein also blocks the host IFN system.

Recently, data were presented indicating that the amino-terminal domain of the measles virus (a morbilivirus) P protein is a "natively unfolded protein" (25). It was also predicted that the amino-terminal region of the P proteins of other morbilliviruses, of the paramyxovirus Sendai virus, and of the rhabdovirus vesicular stomatitis virus are also natively unfolded (25). This finding is in contrast to findings for the amino-terminal domains of the P proteins of the rubulaviruses, a group that includes NDV, which are predicted to be folded (25). When the amino-terminal domain common to the Nipah virus P, V, and W is analyzed in the same way, it is also predicted to be a natively unfolded protein (data not shown). Any connection between this property and the ability to counteract the IFN response remains to be determined.

The identification of the NDV V and the Nipah virus C, V, and W proteins as having IFN-antagonist activity suggests that they are important virulence factors. A role for the NDV V protein in virulence has already been demonstrated. A recombinant NDV with an editing site mutation such that the virus produces 20-fold less V protein than wild-type NDV was highly attenuated in chicken embryos (39). An NDV completely unable to produce V, but presumably able to produce a truncated "amino terminus only" form of V, was highly impaired in tissue culture and unable to replicate in 10-day-old embryonated chicken eggs (39). Other studies provide additional evidence for the importance of the V protein in the virulence of several other paramyxoviruses. Mutations truncating the V protein before the unique carboxy terminus or mutations affecting the ability of V protein to bind zinc attenuated Sendai virus in mice (24, 26). Mutations preventing expression of the unique domains of either V or D (produced from a +2G transcript) had little effect on hPIV-3 replication, either in tissue culture or in vivo. However, mutation of both the V and the D ORFs did yield a modest attenuation phenotype in vivo in a hamster model and in a monkey model (13). The relationship between the IFN system and the various attenuation phenotypes seen with these particular mutant viruses remains to be determined. In contrast, there is a clear correlation between virulence and the anti-IFN function of the Sendai virus C proteins (13, 20).

The NDV-GFP-based assay used to identify IFN-antagonist functions for NDV and Nipah virus proteins is similar to our previously described assay which used a mutant influenza virus, influenza delNS1 virus, which lacks the influenza virus IFN-antagonist NS1 protein (2). In this previously described assay, we found that transfection of MDCK cells with plasmids encoding IFN antagonists greatly enhanced growth of the mutant influenza virus (2). The NDV-GFP-based assay should be complementary to the influenza delNS1 virus-based assay. For example, the use of different viruses with different host ranges may allow a wider range of cell lines to be used when screening for IFN antagonists. Such assays will likely provide new insights into viral pathogenesis. Previous studies on NDV and other paramyxoviruses suggest that these anti-IFN functions play important roles in viral pathogenesis. The observations made in the present report regarding Nipah virus may therefore be of particular interest because Nipah virus is a highly lethal, emerging virus of concern as a potential agent of bioterrorism (9, 14, 34).

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